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TITLE: Molecular Mechanisms and Treatment Strategies for Obesity-Associated Coronary Artery Disease, an Imminent Military Epidemic

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14. ABSTRACT

There is an epidemic of obesity in the military. Obesity leads to type 2 diabetes, the most dangerous consequence of which is atherothrombotic vascular disease. We have made major progress on the key Tasks over the last year. We have gained more in-depth understanding on how the AngII targets CaMKII and NADPH oxidase trigger apoptosis in ER-stressed macrophages. Our knowledge of how PPARs and obesity affect advanced plaque progression was expanded into the areas of monocyte/macrophage subsets and efferocytosis. The mechanism of obesity-associated adipokines was advanced by showing that LPS, as a model of adiponectin-LPS complex, can suppress a pro-apoptotic branch of the UPR *in vivo* by the exact same mechanisms elucidated *in vitro*. Moreover, we found that another obesity-associated adipokine—eNampt—may promote macrophage-associated disease processes in obese subjects. Finally, we showed that a specific molecular event that could promote plaque necrosis and likely occurs in obesity—cleavage of the efferocytosis receptor Mertk—occurs in advanced human plaques. In summary, we have made substantial progress in understanding how obesity leads to accelerated heart disease at a molecular-cellular level. Further work in these areas during Year #4 is likely to suggest novel therapeutic targets to prevent obesity-associated vascular disease in military personnel and in the general public.

15. SUBJECT TERMS
obesity, diabetes, insulin resistance, heart disease, atherosclerosis, macrophages, apoptosis, unfolded protein response, PPAR, adiponectin, angiotensin 2

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INTRODUCTION:

As described in detail in the original grant application, there is an epidemic of obesity in the military. Obesity leads to insulin resistance syndromes, notably metabolic syndrome and type 2 diabetes. The major cause of death in people with insulin resistance syndromes is atherothrombotic vascular disease, including acute myocardial infarction, sudden death, and stroke. Therefore, when retired military personnel and their families reach middle age, there will be an epidemic of obesity-related vascular disease. This will result in the loss of senior personnel and the expertise they contribute to the military. Moreover, the economic burden of cardiovascular disease in active and retired personnel and their families on the military will be enormous. The impact of this trend is being felt now in the military but will accelerate to a very high level over the next 10-20 years if the current trends are left unchecked. In this context, the overall objective of the proposal is to understand at a cellular and molecular level how obesity/insulin resistance promotes atherothrombotic vascular disease. Accomplishing this goal will suggest new targets for drug therapy, which would greatly benefit both the military and the general population. The emphasis of the work is on a key event in advanced atherosclerosis that leads to acute vascular events, namely, advanced lesional macrophage death. Macrophages are the major cell type in atherosclerotic lesions, and when they die and the cell corpses are not rapidly cleared by neighboring phagocytes ("defective efferocytosis"), necrosis ensues. Plaque necrosis, in turn, promotes plaque disruption and exposure of thrombogenic material. The newly exposed thrombogenic material triggers platelet aggregation (thrombosis), which can acutely obstruct the arterial lumen and cause tissue death (infarction). In the heart, this series of events leads to myocardial infarction and sudden death, and in the brain the consequence is stroke. **Thus, the overall focus of this proposal is advanced lesional macrophage death and defective efferocytosis, and we have excellent progress in the third year of funding in understanding how obesity/insulin resistance can promote these processes.**

BODY:

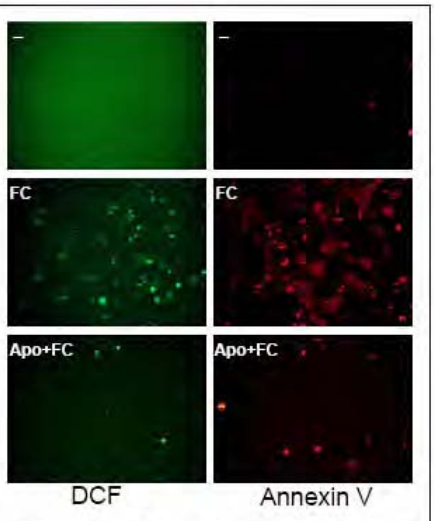
I. Studies related to angiotensin-II (AngII) (Tasks 1-3)

We have continued to make major progress in understanding the fundamental mechanisms of advanced lesional macrophage death that may be impacted by AngII, with an emphasis on our ER stress/unfolded protein response (UPR) model of apoptosis. Moreover, we have explored an exciting new hypothesis that states that the effects of AngII on ER stress-induced macrophage depends upon the relative adaptor system used by the AngII receptor.

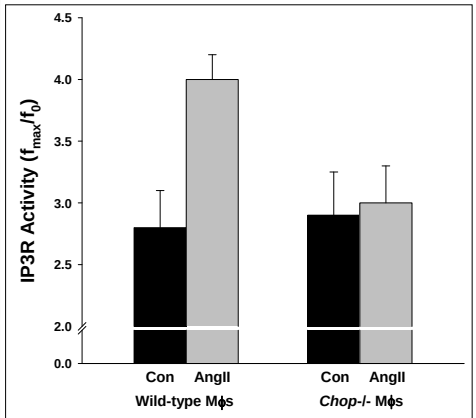
A. AngII target #1: CaMKII—Fern *et al*. (1) were the first to show that AngII activates CaMKII using an adrenal glomerulosa cell model. Most importantly, Palomeque *et al*. (2) reported that AngII causes apoptosis in cardiac myocytes through a signaling pathway that is dependent on CaMKII signaling. The authors confirmed that AngII treatment of the cells resulted in activation of CaMKII. In a paper that has now been published in *Circulation* (**Lim et al.; see Appendix**), we showed for the first time that calcium/calmodulin-dependent protein kinase II (CaMKII) was critical for ER stress-induced apoptosis in macrophages. In a study that will be submitted shortly (Timmins *et al*.—see publication list), we first showed that Fas death receptor mRNA and protein are markedly induced in an ER stress-dependent and

calcium-dependent manner. The mechanism involves activation of an ER stress—CaMKII—JNK—Fas pathway. Remarkably, CaMKII was also responsible for two key processes involved in mitochondrial-dependent apoptosis: release of mitochondrial cytochrome c and loss of mitochondrial membrane potential. CaMKII triggered these events by promoting the uptake of calcium into the mitochondria, and there was a marked increase in CaMKII association with mitochondria after the induction of ER stress. These data support an integrated model in which CaMKII serves as a unifying link between ER stress and *both* the Fas and mitochondrial apoptotic pathways. In addition, the study has revealed a novel pro-apoptotic function of CaMKII, namely, promotion of mitochondrial calcium uptake. These findings provide a molecular link between AngII and ER stress-induced apoptosis and raise the possibility that CaMKII inhibitors might complement AngII inhibitors in preventing apoptosis in pathological settings involving ER stress-induced apoptosis.

B. AngII target #2: NADPH oxidase—Griendling *et al*. (3) reported that AngII stimulates NADPH oxidase in vascular smooth muscle cells. AngII-induced superoxide formation was suppressed by an NADPH oxidase inhibitor. Lodha *et al*. (4) showed that an AngII-NADPH oxidase-ROS (reactive oxygen species) pathway was involved in AngII-induced apoptosis in mouse mesangial cells. AngII also activates NADPH oxidase in macrophages (5), and the type of NADPH oxidase in macrophages (Nox2) is particularly sensitive to activation by AngII (6), but no link to apoptosis was made in this cell type. In recent work in our laboratory, we have shown that macrophages treated with the NADPH oxidase inhibitor apocynin and macrophages from Nox2-deficient mice are markedly resistant to ER stress-induced ROS and apoptosis (**see figure for apocynin data**). The mechanism appears to be that NADPH oxidase-generated ROS amplifies the ER stress response, including calcium release. The calcium release effect seems to rely on ROS-mediated activation of the ER calcium release protein, IP3R. Given that AngII both activates NADPH oxidase and leads to calcium release, the pathway we described could be a direct target of AngII. **In fact, we have shown for the first time that AngII activates IP3R-mediated calcium release by a pathway that depends on the UPR effector CHOP (figure).**

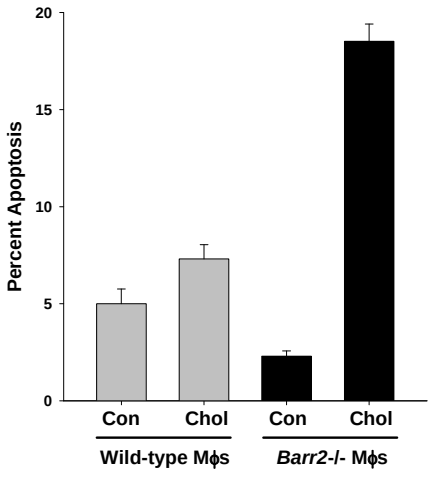


Inhibition of NADPH oxidase by apocynin blocks ER stress–induced macrophage ROS and apoptosis. The images show monolayers of control and treated macrophages that were stained for ROS and apoptosis. FC = free cholesterol loading as a means to trigger ER stress-induced apoptosis. Apo = apocynin. DCF = fluorescent marker of ROS. Annexin V = assay for apoptosis. Similar data were obtained using NADPH oxidase-negative (*Nox2-/-*) macrophages instead of apocynin.



IP3 receptor activation by AngII requires the UPR effector CHOP. Wild-type or *Chop-/-* macrophages were incubated with or without 200 nM AngII for 16 hrs. The cells were then assayed for IP3R activity by stimulation with 10 μM ATP followed by measurement of fluorescence intensity of cytosolic Fura-2 compared to baseline fluorescence (f_{max}/f_0).

C. AngII receptor adaptors: beta-arrestin 2—The net effect of AngII on macrophage apoptosis likely depends on the balance between cell death pathways and cell survival pathways (7). The type I AngII receptor can signal through the adaptor β-arrestin, which is known to activate ERK (8). ERK is a survival pathway in ER-stressed macrophages (unpublished data). However, the AngII receptor can also signal in an arrestin-independent manner, leading to the processes described above, *e.g.*, calcium release and NADPH oxidase activation (8), which would likely enhance ER stress-induced macrophage apoptosis. To begin to explore this idea, we first determined β-arrestin expression in macrophages. We found that these cells express primarily β-arrestin 2 but also some β-arrestin 1. In a preliminary experiment, we found that macrophages from β-arrestin 2 knockout mice, obtained from Dr. Robert Lefkowitz at Duke University, were **much more susceptible to ER stress-induced apoptosis, as suggested by our hypothesis (figure)**. Ongoing studies are looking at the effect of both β-arrestin 1 and 2 on the ability of AngII to promote apoptosis in macrophages.



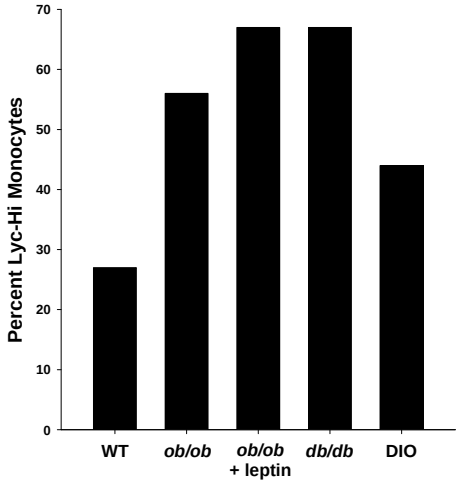
Absence of β-arrestin 2 markedly enhances ER stress-induced macrophage apoptosis. Con = control (non-ER-stressed macrophages); Chol = cholesterol loaded macrophages as a model of ER stress; Barr2-/- = β-arrestin 2 knockout. Apoptosis was assayed by annexin V staining.

D. Implications—AngII is a major risk factor for acute coronary syndromes, which are precipitated by vulnerable plaques associated with macrophage death. The above findings are showing clear areas of relationships between specific mechanisms of plaque macrophage death and AngII signaling. However, the situation is extremely complex. First, the balance of ER stress signaling and AngII signaling may determine the relative importance of each pathway. Second, AngII signaling is dichotomous and can trigger cell survival pathways. Indeed, ER stress might effect the particular pathways used by the AngII receptor. Our new findings on the role of β-arrestin 2 in ER stress-induced macrophage death may open up a new window of opportunity to sort out these points, both in mechanistic studies *in vitro* and in atherosclerosis studies *in vivo*. We are currently expanding our colony of β-arrestin 2 knockout mice, and we are also developing siRNA reagents for the β-arrestins. Mechanistic studies are ongoing to address the aforementioned ideas, and future *in vivo* studies will be started as soon as the mouse colony expands.

II. PPAR studies (related to Tasks 3-5)

Plaque necrosis is caused by the combination of macrophage apoptosis and defective phagocytic clearance ("efferocytosis") of the apoptotic cells (9). In a recent study in our laboratory (Thorp, E., Cui, D., Kuriakose, G., and Tabas, I. (2008) Mutation of the Mertk receptor promotes apoptotic cell accumulation and plaque necrosis in advanced atherosclerotic lesions of apolipoprotein E-deficient mice. *Arterio. Thromb. Vasc. Biol.* 28:1421-8), we showed a direct molecular link between a key efferocytosis receptor—Mertk—and both efferocytosis and plaque necrosis in advanced atherosclerotic lesions. It turns out that advanced lesions have several different types of macrophages, often referred to as classically activated inflammatory macrophages and alternatively activated resolution macrophages. In general, the inflammatory macrophages originate from a subset of blood-borne monocytes that express high levels of a marker called Lyc (Lyc-high monocytes), and

vice versa for the alternatively activated macrophages (Lyc-low monocytes). PPARs promote the formation of alternatively activated macrophages, while obesity promotes the invasion of inflammatory macrophages (10-12). In new work in our laboratory based on this grant, we have begun to explore how PPARs and obesity affect monocyte/macrophage subsets and efferocytosis. Our preliminary data show that macrophages derived from Lyc-low macrophages are better able to dispose of apoptotic cells. Most importantly, obesity leads to a dramatic increase in the ratio of Lyc-high:low monocytes (**figure**)—an effect we think is mediated by decreased activation of PPARγ and PPARδ in obesity. We have also begun to identify different sub-populations of macrophages in advanced atherosclerotic lesions and to determine their relative efferocytotic efficiency. Our hypothesis is the Lyc-high-derived macrophages will be increased in advanced lesions and will have relatively poor efferocytic capacity. Finally, we are setting up mouse models of PPAR activation and depletion to test our ideas in a causative manner.



Obesity is associated with an elevated percentage of Lyc^{hi}:Lyc^{lo} monocytes. Peripheral blood monocytes from thin wild-type (WT) mice and obese ob/ob, db/db, and diet-induced obese mice (DIO) were assayed by FACS for Lyc. Note that addition of leptin to ob/ob mice, which are leptin-deficient, did not reverse the effect, indicating that leptin is not involved.

III. Adiponectin/adipokine studies (Task 6)

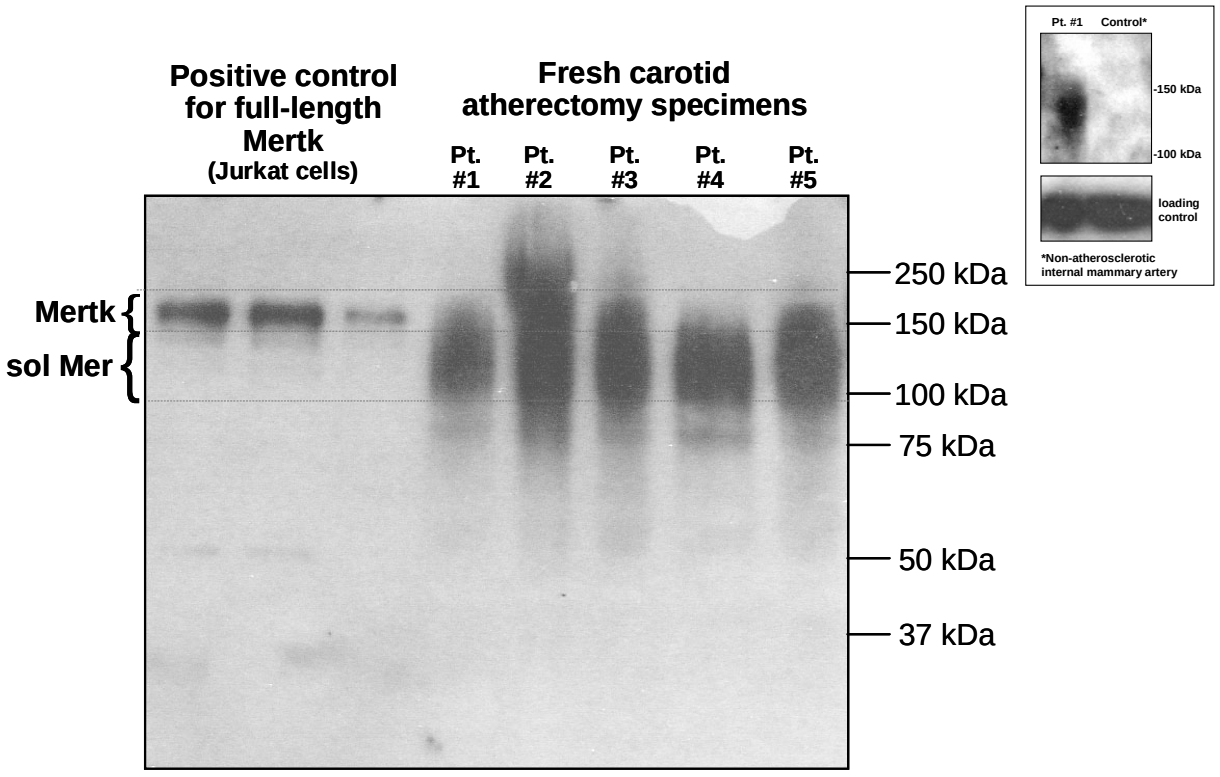
As explained last year, we have evidence that adiponectin may signal partially through a bound LPS molecule in adiponectin's hydrophobic core. The adiponectin-LPS complex has a profound effect on inducing macrophage survival, which could explain some of the beneficial effects of adiponectin and weight loss on CAD. In particular, as explained last year, the complex suppresses that ATF4-CHOP branch of the UPR, which helps explains its pro-survival effect in macrophages. **Throughout 2008, we have made major progress in obtaining *in vivo* data to support our mechanistic findings (Woo *et al*., manuscript in preparation).** We found that pre-treating mice with LPS (as a model of adiponectin-LPS complex) protected the animals from subsequent UPR-induced apoptosis and organ damage in the kidney, liver, and spleen. Moreover, the mechanisms elucidated *in vitro* are exactly those that operate *in vivo*. Further work in year #4 will apply these findings to adiponectin and obesity.

We have expanded Task 6 by including the study of another adipose-generated and obesity-related adipocytokine called extracellular nicotinamide phosphoribosyltransferase (eNampt; aka PBEF, visfatin). Although the emphasis of this grant has been on macrophage apoptosis, excess macrophage survival can be detrimental in obesity, because macrophages promote *early* atherogenesis (as opposed to advanced plaque necrosis), insulin resistance, and tumors—all obesity-associated diseases. As described in **Li *et al.* in the Appendix**, adipocytes and other cells secrete eNampt, and plasma levels of eNampt increase in obesity. We tested the hypothesis that eNampt could promote cell survival in macrophages subjected to ER stress, a process associated with obesity and obesity-associated diseases. We show that eNampt potently blocks macrophage apoptosis induced by a number of ER stressors. The mechanism involves a two-step sequential process: rapid induction of interleukin 6 (IL-6) secretion, followed by IL 6-mediated autocrine/paracrine activation of the pro-survival signal

transducer STAT3. The ability of eNampt to trigger this IL 6-/STAT3 cell-survival pathway did not depend on the presence of the Nampt enzymatic substrate nicotinamide in the medium; could not be mimicked by the Nampt enzymatic product nicotinamide mononucleotide (NMN); was not blocked by the Nampt enzyme inhibitor FK866; and showed no correlation with enzyme activity in a series of site-directed mutant Nampt proteins. Thus, eNampt protects macrophages from ER stress-induced apoptosis by activating an IL-6/STAT3 signaling pathway via a non-enzymatic mechanism. These data suggest a novel action and mechanism of eNampt that could affect the balance of macrophage survival and death in the setting of obesity, which in turn could play important roles in obesity-associated diseases. In particular, our working hypothesis is that those obesity-associated conditions in which macrophages are detrimental, including early atherogenesis, white adipose tissue-mediated inflammation and insulin resistance, and tumors promoted by tumor-associated macrophages (TAMs), could be exacerbated by the high levels of eNampt in obesity. We have accumulated mouse models to explore this idea *in vivo* in the following year.

IV. Studies in advanced human atherosclerotic lesions (Task 7)

Our new work in this arena has focused on the important concept described above, namely, the role of defective efferocytosis in advanced plaque necrosis. Given the important role of Mertk in this efferocytosis and the fact that Mertk can be cleaved to soluble Mer, thus compromising efferocytosis (13), we asked the question whether soluble Mer is found in advanced human lesions. We found a remarkably high level of soluble Mer in advanced human lesions but not in uninvolved human arteries (**figure**). Given that advanced lesion are known to have defective efferocytosis, our new data provide a possible explanation.



Soluble Mer is present in advanced human plaques. Western blot using an anti-human Mertk antibody. The predicted migration of full-length and soluble Mertk (sol Mer) are indicated on the left. Soluble Mer runs as a diffuse band due to variations in protein glycosylation. Jurkat cells were used as a control for uncleaved Mertk. Specimens from 5 patients are shown. The inset shows a non-atherosclerotic artery as a negative control.

Importantly, macrophage inflammation is an essential part of obesity, and Mertk cleavage is induced by macrophage inflammation (13). Over the next year, we will test this hypothesis in vivo using mice expressing non-cleavable Mer.

KEY RESEARCH ACCOMPLISHMENTS IN YEARS 1-3 AND PLANS FOR YEAR 4:

<table>
<tr><th>Task #</th><th>Year 1</th><th>Year 2</th><th>Year 3</th><th>Planned Year 4</th></tr>
<tr><td>1</td><td rowspan="2">• Initiation of key signaling studies that we reasoned would be critical for understanding the role of AngII in Mϕ apoptosis.</td><td rowspan="2">• Elucidation of the key signaling pathways involved in the ER stress-PRR model of apoptosis, with an emphasis on those pathways known to be direct targets of AngII, namely, calcium-CaMKII-NADPH oxidase-ROS. This work resulted in a high-profile manuscript recently accepted by Circulation.</td><td rowspan="2">• AngII target #1: In-depth mechanistic work in on how calcium is released to activate CaMKII
• AngII target #2: New insight into how NADPH oxidase promotes apoptosis
• New data how the AngII receptor adaptor β-arrestin 2 can influence the balance between apoptosis and cell survival</td><td rowspan="2">• We will continue to expand our understanding of how the AngII targets CaMKII and NADPH oxidase, promote apoptosis and how they are further affected by AngII signaling
• The effect of β-arrestins on pro-apoptotic vs. pro-survival signaling by AngII will be explored both in vitro and in vivo. For the in vivo studies we will relate to obesity by using the ob/ob model.</td></tr>
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<tr><td>3</td><td rowspan="3">• Elucidation of the role of TZDs on advanced lesional Mϕ death.</td><td rowspan="2">• The TZD study was refined with more in-depth mechanism and completion of in vivo studies, resulting in submission and publication by Circulation.</td><td rowspan="2">• Discovery of how PPARs and obesity can effect monocyte/macrophage subsets
• New data on how these monocyte/macrophage subsets affect the efficiency of efferocytosis</td><td rowspan="3">• We are using mice treated with PPAR activators and mice deficient in PPARs to test the hypothesis that PPAR has effects on monocyte/macrophage subsets
• We will continue to explore how different subsets of macrophages in advanced plaques affect apoptosis and efferocytosis and how PPARs affect these processes</td></tr>
<tr><td>4</td></tr>
<tr><td>5</td></tr>
<tr><td>6</td><td>• Mechanistic studies on how adiponectin suppresses the UPR.</td><td>• Elucidation both in vitro and in vivo that the key UPR-suppressive action of adiponectin is carried out by an adiponectin-LPS complex (holo-adiponectin) through a unique TLR-TRIF/TRAM pathway.</td><td>• New comprehensive data that the adiponectin-LPS UPR suppressive pathway functions in vivo
• New study on a 2nd adipokine—eNampt—showing how it promotes macrophage survival and thus may contribute to obesity related macrophage-induced</td><td>• We have crossed adiponectin knockout mice onto the Ldlr-/- background and will explore the effect on macrophage apoptosis and plaque necrosis
• We will determine whether eNampt promotes macrophage survival and macrophage-associated diseases in obese mice</td></tr>
</table>

			diseases	
7	• Identification of key downstream UPR effectors as candidates to explore in human lesions.	• First demonstration that a key pro-apoptotic signaling molecule downstream of the UPR—Ser-P-STAT1—is expressed in advanced but not early human coronary artery lesions. (Myoishi et al. reported that advanced human lesions express UPR markers, which was correlated with apoptosis and plaque vulnerability.)	• First demonstration that soluble Mer, a marker of defective efferocytosis, is present in advanced human plaques	• We will continue to probe human lesions for the key signaling molecules of UPR-induced apoptosis, including CaMKII and NADPH oxidase, and effective efferocytosis, including the enzyme responsible for cleaving Mertk

REPORTABLE OUTCOMES:

- Original Publications

Lim, W., Timmins, J., Seimon, T.A., Sadler, A., Kolodgie, F., Virmani, R., Schindler, C., and Tabas, I. (2008) A pathway involving calcium/calmodulin-dependent protein kinase II and serine-phosphorylated Stat1 is critical for endoplasmic reticulum stress-dependent macrophage apoptosis. A new component of the multi-hit model of macrophage death relevant to advanced atherosclerosis. *Circulation* 117:940-951.

Li, Y., Zhang, Y., Dorweiler, B., Cui, D., Wang, T., Woo, C.W., Wolberger, C., Imai, S., Tabas, I. (2008) Extracellular Nampt protects macrophages from ER stress-induced apoptosis via a non-enzymatic interleukin-6/STAT3 signaling mechanism. *J. Biol. Chem.*, In press.

- Manuscripts close to submission

Timmins, J., Seimon, T., Ozcan, L., Li, G., Tabas, I. (2008) Calcium/calmodulin-dependent protein kinase II links endoplasmic reticulum stress with Fas and mitochondrial apoptosis pathways. Manuscript in preparation.

Woo, C.W., Cui, D., Arrelano, J., Dorweiler,B., Harding, H., Fitzgerald, K.A., Ron, D., and Tabas, I. (2008) Adaptive suppression of the ATF4-CHOP branch of the unfolded protein response by toll-like receptor signaling. Manuscript in preparation.

- Reviews

Tabas, I. (2008) Macrophage apoptosis in atherosclerosis: consequences on plaque progression and the role of endoplasmic reticulum stress. *Antioxid. Redox Signal.* In press.

Seimon, T. and Tabas, I. (2008) Mechanisms and consequences of macrophage apoptosis in atherosclerosis. *J. Lipid Res.*, In press.

- Post-doctoral fellows are assembling their data on LPS-mediated UPR suppression, the role of CaMKII in apoptosis, and the role of NADPH oxidase in apoptosis

- Oral presentations on the material described in this progress report

- Post-doctoral training related to the aforementioned projects in the Tabas laboratory

CONCLUSIONS:

During year #3, we have gained more in-depth understanding on how the AngII targets CaMKII and NADPH oxidase trigger apoptosis in ER stressed macrophages. We also have begun to accumulate evidence in support of our hypothesis that the overall effect of AngII on macrophages will depend on the relative utilization of β-arrestin 2 cell survival pathway and a non-arrestin pro-apoptotic pathway. The pro-apoptotic pathway appears to feed into the calcium-CaMKII pathway via AngII-mediated activation of IP3R activity. Our knowledge of how PPARs and obesity might affect advanced plaque progression was expanded into the areas of monocyte/macrophage subsets and efferocytosis. Our working hypothesis is that PPARs promote, and obesity inhibits, the generation of macrophages that are very efficient efferocytes. The mechanism of obesity-associated adipokines was advanced by showing that LPS, as a model of adiponectin-LPS complex, can suppress a pro-apoptotic branch of the UPR *in vivo* by the exact same mechanisms elucidated *in vitro*. Moreover, we made a novel discovery related to another obesity-associated adipokine—eNampt—which forms the basis of a hypothesis related to how obesity promotes macrophage-associated disease processes. Finally, we showed for the first time that a specific anti-efferocytic molecular event that could promote plaque necrosis—cleavage of the efferocytosis receptor Mertk—occurs in advanced human plaques. Mertk cleavage is promoted by macrophage inflammation, which is key aspect of obesity. In summary, we have made substantial progress in understanding how obesity leads to accelerated heart disease at a molecular-cellular level by the elucidation of novel pathways of macrophage apoptosis in advanced atherosclerosis. Continuing work on the Tasks of this grant during Year #4 will hopefully suggest new therapeutic approaches to this evolving epidemic in the military and in the general society.

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13. **Sather,S., Kenyon,K.D., Lefkowitz,J.B., Liang,X., Varnum,B.C., Henson,P.M., and Graham,D.K. 2007. A soluble form of the Mer receptor tyrosine kinase inhibits macrophage clearance of apoptotic cells and platelet aggregation. *Blood* 109:1026-1033.**

APPENDICES:

- Lim, W., Timmins, J., Seimon, T.A., Sadler, A., Kolodgie, F., Virmani, R., Schindler, C., and Tabas, I. (2008) *Circulation* 117:940-951.

- Li, Y., Zhang, Y., Dorweiler, B., Cui, D., Wang, T., Woo, C.W., Wolberger, C., Imai, S., Tabas, I. (2008) *J. Biol. Chem.*, In press.

- Tabas, I. (2008) *Antioxid. Redox Signal.* In press.

- Seimon, T. and Tabas, I. (2008) *J. Lipid Res.*, In press.

Signal Transducer and Activator of Transcription-1 Is Critical for Apoptosis in Macrophages Subjected to Endoplasmic Reticulum Stress In Vitro and in Advanced Atherosclerotic Lesions In Vivo

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Signal Transducer and Activator of Transcription-1 Is Critical for Apoptosis in Macrophages Subjected to Endoplasmic Reticulum Stress In Vitro and in Advanced Atherosclerotic Lesions In Vivo

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Background—Macrophage apoptosis is a critical process in the formation of necrotic cores in vulnerable atherosclerotic plaques. In vitro and in vivo data suggest that macrophage apoptosis in advanced atheromata may be triggered by a combination of endoplasmic reticulum stress and engagement of the type A scavenger receptor, which together induce death through a rise in cytosolic calcium and activation of toll-like receptor-4.

Methods and Results—Using both primary peritoneal macrophages and studies in advanced atheromata in vivo, we introduce signal transducer and activator of transcription-1 (STAT1) as a critical and necessary component of endoplasmic reticulum stress/type A scavenger receptor–induced macrophage apoptosis. We show that STAT1 is serine phosphorylated in macrophages subjected to type A scavenger receptor ligands and endoplasmic reticulum stress in a manner requiring cytosolic calcium, calcium/calmodulin-dependent protein kinase II, and toll-like receptor-4. Remarkably, apoptosis was inhibited by ≈80% to 90% ($P<0.05$) by STAT1 deficiency or calcium/calmodulin-dependent protein kinase II inhibition. In vivo, nuclear Ser-P-STAT1 was found in macrophage-rich regions of advanced murine and human atheromata. Most important, macrophage apoptosis was decreased by 61% ($P=0.034$) and plaque necrosis by 34% ($P=0.02$) in the plaques of fat-fed low density lipoprotein receptor null *Ldlr*$^{-/-}$ mice transplanted with *Stat1*$^{-/-}$ bone marrow.

Conclusions—STAT1 is critical for endoplasmic reticulum stress/type A scavenger receptor–induced apoptosis in primary tissue macrophages and in macrophage apoptosis in advanced atheromata. These findings suggest a potentially important role for STAT1-mediated macrophage apoptosis in atherosclerotic plaque progression. **(*Circulation*. 2008; 117:940-951.)**

Key Words: apoptosis ■ atherosclerosis ■ cholesterol ■ macrophage ■ plaque

In advanced atherosclerosis, death of macrophages in the setting of defective phagocytic clearance of apoptotic cells contributes to the development of plaque necrosis.[1,2] Plaque necrosis, in turn, is thought to promote plaque disruption and arterial thrombosis, which are the proximate causes of acute cardiovascular events.[1–3] Our laboratory established an important principle of advanced lesional macrophage death, namely involvement of the endoplasmic reticulum (ER) stress pathway known as the unfolded protein response (UPR).[4,5] Some laboratories have discovered important evidence that the UPR is activated in intimal cells, including macrophages, in advanced murine and human plaques.[6–9] In particular, Myoishi et al[9] recently showed a dramatic rise in UPR markers, including the transcription factor CHOP (GADD153), and intimal cell apoptosis in autopsy specimens from humans with vulnerable and ruptured plaques but not stable lesions and in atherectomy specimens from humans with unstable angina but not stable angina. Although the UPR is primarily an ER repair pathway, a branch of the UPR involving the effector CHOP can trigger apoptosis when the cell senses that repair is no longer possible.[4,10] In terms of causation, we have shown that advanced lesional macrophage death and plaque necrosis are decreased in atherosclerotic apolipoprotein E–deficient (*Apoe*$^{-/-}$) mice in the setting of ER stress prevention[5] or CHOP deficiency (Edward Thorp, PhD; Gang Li, PhD; George Kuriakose, MSc; David Ron, MD; and I.T., unpublished data, 2007).

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The online-only Data Supplement, consisting of Methods and a figure, can be found with this article at http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.107.711275/DC1.

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Our work on the UPR began with a model of advanced lesional macrophage death that is present in advanced plaques, namely intracellular accumulation of lipoprotein-derived free cholesterol (FC).[11] FC enrichment of macrophages, like many ER stressors, activates the UPR through depletion of ER luminal calcium.[12,13] Since then, mechanistic studies have led to a broader concept of advanced lesional macrophage death beyond the FC model. These studies have shown that any combination of inducers of ER stress and ligands for the macrophage type A scavenger receptor (SRA), both of which are expressed prominently in advanced lesions, triggers macrophage apoptosis.[14,15] Macrophage SRA recognizes a number of lesional molecules and atherogenic lipoproteins, including those used to enrich macrophages with cholesterol in the FC model.[16] The SRA also is a pattern recognition receptor of the innate immune system, and endotoxin-free SRA ligands activate other pattern recognition receptors, notably toll-like receptor-4 (TLR4).[15,17,18] In this context, our studies have shown that SRA ligands trigger 2 critical proapoptotic events in ER-stressed macrophages: TLR4-mediated activation of a proapoptotic MyD88 pathway[15] and SRA-mediated suppression of a prosurvival TLR4-TRIF-interferon (IFN)-β pathway.[14,15]

In this report, we show that apoptosis of ER-stressed macrophages also requires signal transducer and activator of transcription-1 (STAT1) and calcium/calmodulin-dependent protein kinase II (CaMKII) in a process involving cytosolic calcium and TLR4. Most important, we provide evidence that activated STAT1 is present in atheromata and that lesional macrophage apoptosis is suppressed in the setting of STAT1 deficiency.

Methods

See the online-only Data Supplement for expanded Methods.

Assay of Macrophage Apoptosis

Midstage and late-stage apoptosis in peritoneal macrophages was assayed by annexin V and propidium iodine staining, respectively, with the Vybrant Apoptosis Assay Kit No. 2 (Molecular Probes, Carlsbad, Calif). At the end of incubation, the macrophages were gently washed once with PBS and incubated for 15 minutes at room temperature with 120 μL annexin-binding buffer (25 mmol/L HEPES, 140 mmol/L NaCl, 1 mmol/L EDTA, pH 7.4, 0.1% BSA) containing 10 μL Alexa Fluor 488–conjugated annexin V and 1 μL of 100-μg/mL propidium iodine. The staining mixture was then removed and replaced with 120 μL annexin-binding buffer. The cells were viewed immediately at room temperature with an Olympus IX-70 inverted fluorescent microscope equipped with filters appropriate for fluorescein and rhodamine, and images were obtained with a Cool Snap charge-coupled device camera (RS Photometrics, Tucson, Ariz) equipped with imaging software from Roper Scientific (Trenton, NJ). Three fields of cells ($\approx$650 cells per field) were photographed for each condition, and the number of annexin V/propidium iodine–positive cells in each field was counted and expressed as a percent of the total number of cells.

Bone Marrow Transplantation

Ten-week-old female low-density lipoprotein receptor null *Ldlr*$^{-/-}$ mice were lethally irradiated with 10 Gy from a cesium γ source 4 to 6 hours before transplantation. Bone marrow cells were collected from the femurs and tibias of donor *Stat1*$^{-/-}$ or *Stat1*$^{+/+}$ mice by flushing with sterile medium (RPMI 1640, 2% FBS, 10 U/mL heparin, 50 U/mL penicillin, 50 μg/mL streptomycin). The bone marrow cells were washed extensively and resuspended in RPMI medium containing 20 mmol/L HEPES, 50 U/mL penicillin, and 50 μg/mL streptomycin. Each recipient mouse was injected with 5×10^6 bone marrow cells through the tail vein. The mice were given acidified water containing 100 mg neomycin and 10 mg polymyxin B sulfate 1 week before and 2 weeks after transplantation. Six weeks after transplantation, the mice were fed a "Western-type" diet (21% anhydrous milk fat and 0.15% cholesterol, TD88137, Harlan-Teklad) for 10 or 12 weeks.

Atherosclerotic Lesion Analysis

On the day of the analysis, food was removed from the cages in the morning, and the mice were fasted for 8 hours. The animals were then anesthetized with isoflurane, and blood was withdrawn by cardiac puncture. The heart was then perfused with PBS, and the heart and proximal aorta were harvested. The heart and aorta were perfused ex vivo with PBS and then transferred to 10% buffered formalin, processed, and embedded in paraffin. Starting from the atrial leaflets, serial sections (6 μm thick) were prepared so that every eighth section was stained with Harris hematoxylin and eosin. Atherosclerotic lesions in 6 sections were analyzed in a blinded fashion with a Nikon Labophot-2 microscope (Nikon Instruments Inc, Melville, NY) equipped with a Sony CCD-Iris/RGB color videocamera (Sony Electronics Inc, San Diego, Calif) attached to a computerized imaging system using IMAGE-PRO PLUS 3.0 software (Media Cybernetics, Bethesda, Md). Aortic lesion area was quantified by averaging the lesion areas of the 6 sections. Necrotic areas were defined as those regions of the lesions that lacked nuclei and cytoplasm.

In Situ TdT-Mediated dUTP Nick-End Labeling Assays

Apoptotic cells in the intima of atherosclerotic lesions were detected by the TdT-mediated dUTP nick-end labeling (TUNEL) technique using the TMR red in situ cell death detection kit (Roche Diagnostics, Indianapolis, Ind) and the stringent method of Kockx.[19] Sections of proximal aorta were deparaffinized, rehydrated, and treated with 2 μg/mL proteinase K (Roche) for 30 minutes at 37°C in a humidified chamber. The treated sections were incubated in TdT reaction mixture containing TMR red dUTP for 1 hour at 37°C in a humidified chamber. After washing, genomic DNA was stained with DAPI for 5 minutes at room temperature; the slides then were mounted with coverslips. TUNEL staining was analyzed with an Olympus IX-70 inverted fluorescent microscope equipped with a Cool Snap charge-coupled device camera and imaging software (Roper Scientific). Fluorescent images were captured and analyzed with image Photoshop analysis software (Adobe Systems, San Jose, Calif).

Statistical Analysis

Data are presented as mean$\pm$SEM. Absent error bars in the bar graphs signify SEM values smaller than the graphic symbols. The significance of paired data was determined by Student *t* test. Data with $>$2 groups or $\geq$2 independent variables were analyzed with ANOVA, followed by the Bonferroni post hoc test. Significance is indicated by an asterisk in the figures with an explanation in the figure legends; nonsignificance is indicated by NS in the figures.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

SRA-Induced Apoptosis in ER-Stressed Macrophages Requires STAT1 and Is Preceded by Serine Phosphorylation of STAT1

During the course of another study investigating an ER stress response mediator called interferon-inducible, double-

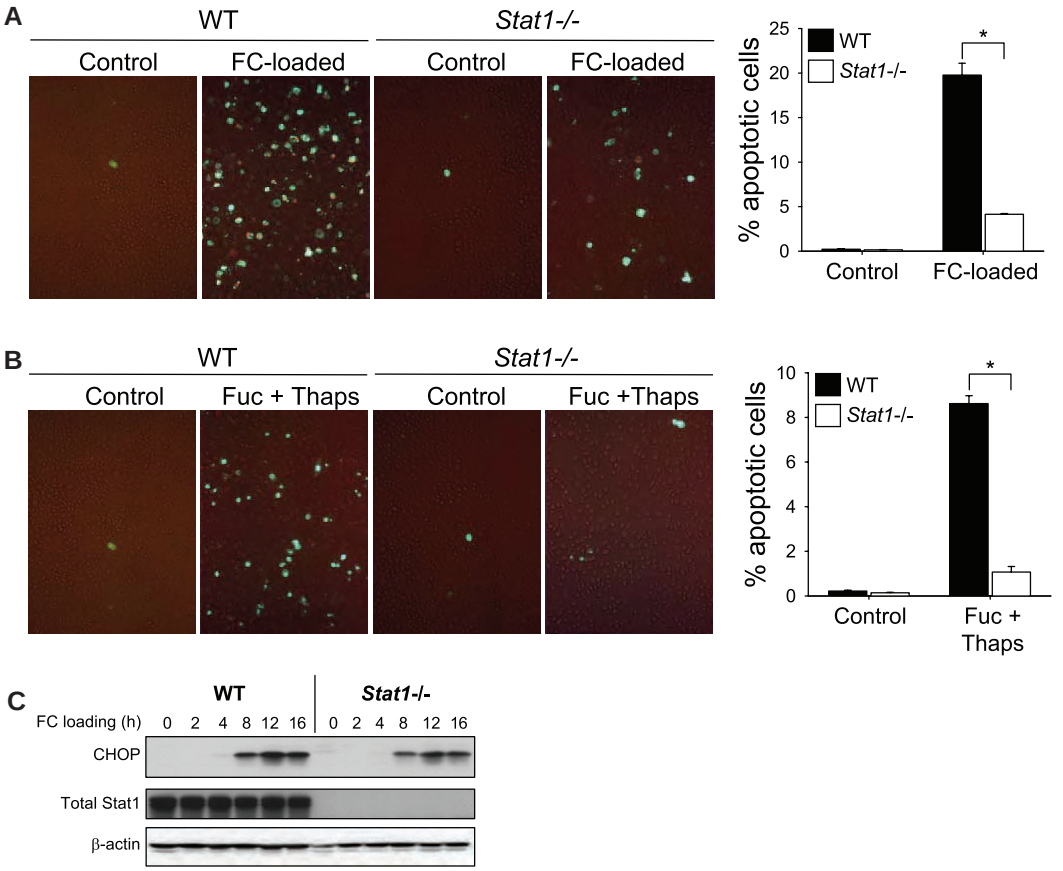


Figure 1. SRA/ER stress–induced macrophage apoptosis requires STAT1. A and B, Peritoneal macrophages from WT or *Stat1*$^{-/-}$ mice were incubated for 17 hours with medium alone (Control) or medium containing acetyl–low-density lipoprotein (LDL) plus the ACAT inhibitor 58035 (FC-loaded) or for 21 hours with medium alone (Control) or medium containing 50 μg/mL fucoidan and 0.5 μmol/L thapsigargin (Fuc+Thaps). Midstage and late-stage apoptosis was assessed by staining with Alexa Fluor 488–conjugated annexin V (green) and propidium iodine (orange), respectively. Representative merged fluorescence and bright-field images and quantitative data from 3 fields of cells for each condition are shown. C, Lysates from WT and *Stat1*$^{-/-}$ macrophages were FC loaded for the indicated times and subjected to immunoblot analysis to detect CHOP, total STAT1, and β-actin. *P=0.001 by Bonferroni after ANOVA.

stranded RNA-regulated elF-2α protein kinase (PKR), we conceived the hypothesis that STAT1, the activity of which is modulated by PKR,[20] may play a role in ER stress–induced macrophage apoptosis. To test this idea, we compared SRA/ER stress–induced apoptosis in peritoneal macrophages from wild-type (WT) versus *Stat1*$^{-/-}$ mice. Confirming our previous work, both intracellular FC enrichment with an SRA-interacting lipoprotein and treatment with the SRA ligand fucoidan plus the UPR activator thapsigargin triggered apoptosis, as indicated by an increase in annexin V staining (Figure 1A and 1B, WT). In contrast, *Stat1*$^{-/-}$ macrophages were markedly protected from apoptosis by both inducers (80% to 90% inhibition of apoptosis; $P<0.05$), indicating an essential role of STAT1 in this model of macrophage apoptosis (Figure 1A and 1B, *Stat1*$^{-/-}$). The decrease in apoptosis in *Stat1*$^{-/-}$ macrophages could not be explained by a decrease in either SRA (not shown) or CHOP induction (Figure 1C).

STAT1 is activated by phosphorylation of Y701 or S727.[21] Y701 phosphorylation is essential for STAT1 dimerization, nuclear translocation, and DNA binding.[21] S727 phosphorylation enhances the transcriptional activity of tyrosine-phosphorylated STAT1 or, in some cases, has been reported to participate in signaling in the absence of Y701 phosphorylation.[21–23] As shown in Figure 2A, FC loading of macrophages induced serine, whereas tyrosine phosphorylation was not detected, and total STAT1 was not increased. In contrast, very little serine phosphorylation was seen in nonloaded or cholesteryl ester–loaded macrophages, which show no or very little evidence of ER stress.[4] As expected, IFNγ induced highly detectable levels of tyrosine phosphorylation and serine phosphorylation of STAT1.[21] Previous work has suggested that nuclear Ser-P-STAT1 can occur through serine phosphorylation of a constitutive pool of nuclear STAT1.[23] We detected STAT1 in nuclear fractions isolated from untreated macrophages, and Ser-P-STAT1 was increased with FC loading. Although total nuclear STAT1 was modestly increased after FC loading, this increase was much less than that seen with IFNγ, which is known to induce STAT1 nuclear translocation[21] (Figure 2B). These data suggest that at least a portion of FC-induced Ser-P-STAT1 occurs through phosphorylation of constitutively nuclear STAT1. It also is possible that at least a portion of the STAT1 was tyrosine phosphorylated but below the limits of detection of our immunoblot assay.

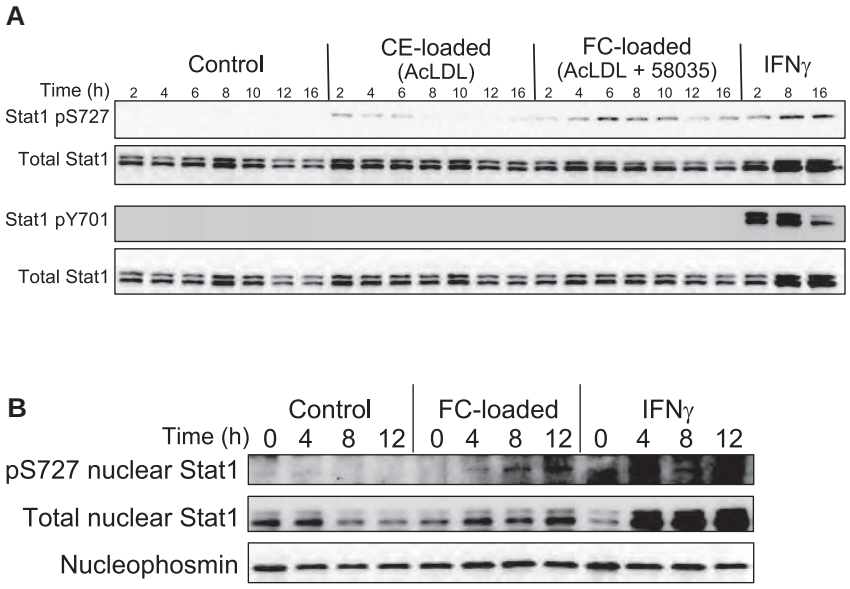


Figure 2. FC loading induces serine but not tyrosine phosphorylation of STAT1. A, Macrophages were incubated for the indicated times with medium alone (Control) or medium containing acetyl-LDL (CE-loaded), acetyl-LDL plus 58035 (FC-loaded), or 100 U/mL IFNγ. Whole-cell lysates were then prepared and subjected to immunoblot analysis to detect phospho–S727 STAT1 (Stat1 pS727), phospho–Y701 STAT1 (Stat1 pY701), and total STAT1. B, Nuclear fractions from control, FC-loaded, and IFNγ-treated macrophages were subjected to immunoblot analysis to detect STAT1 pS727, total STAT1, and the nuclear marker nucleophosmin.

The ability of IFNγ to stimulate both serine and tyrosine phosphorylation of STAT1, the presence of IFNγ in atherosclerotic lesions, and recent evidence that IFNγ promotes advanced plaque progression[24,25] led us to explore the effect of the combination of FC loading and IFNγ treatment on Ser-P-STAT1 and apoptosis. FC-loaded macrophages treated with IFNγ showed an increase in Ser-P-STAT1 that was greater than either condition alone (Figure

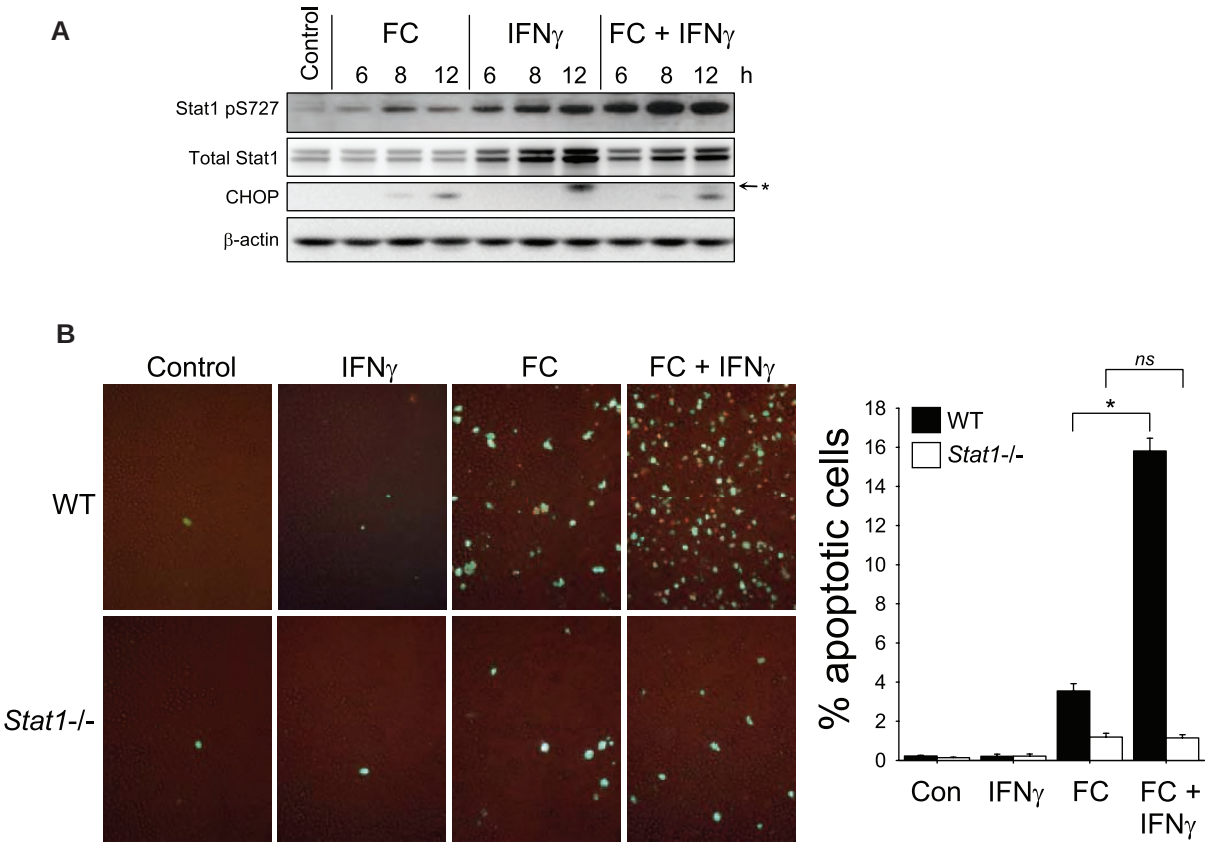


Figure 3. IFNγ enhances FC-induced STAT1 serine phosphorylation and STAT1-dependent FC-induced apoptosis. A, Macrophages were incubated for the times indicated with medium alone (Control) or medium containing acetyl-LDL plus 58035 (FC), 100 U/mL IFNγ, or acetyl-LDL, 58035, and IFNγ (FC+IFNγ). Whole-cell lysates were then prepared and subjected to immunoblot analysis to detect STAT1 pS727, total STAT1, CHOP, and β-actin. In the CHOP blot, a nonspecific band is indicated by the asterisk. B, Macrophages from WT or *Stat1*$^{-/-}$ mice were incubated for 13 hours with medium alone (Control) or medium containing 100 U/mL IFNγ, acetyl-LDL plus 58035 (FC), or acetyl–low-density lipoprotein, 58035, and IFNγ (FC+IFNγ). Apoptosis was assayed and quantified as in Figure 1. *P=0.01 for FC and P=0.001 for FC+IFNγ by Bonferroni after ANOVA.

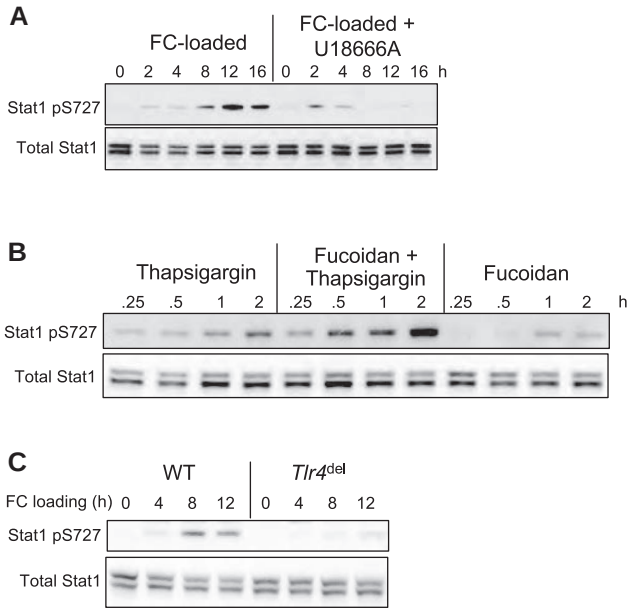


Figure 4. STAT1 serine phosphorylation in SRA-engaged, ER-stressed macrophages is amplified by ER stress and requires TLR4 activation. Whole-cell lysates were subjected to immunoblot analysis to detect STAT1 pS727 and total STAT1 under the following conditions. A, Macrophages were incubated for the times indicated with acetyl-LDL and 58035 (FC-loaded) or acetyl-LDL, 58035, and 1 μmol/L U18666A (FC-loaded+U18666A). B, Macrophages were incubated for the times indicated with 0.5 μmol/L thapsigargin, 50 μg/mL fucoidan plus thapsigargin, or fucoidan alone. C, Macrophages from WT or *Tlr4*^{del} mice were incubated for the times indicated under FC-loading conditions.

3A). Note that IFNγ alone did not induce CHOP, nor did it further increase CHOP in the setting of FC loading. Most important, under conditions in which IFNγ alone induced no apoptosis, IFNγ treatment led to a >5-fold enhancement of FC-induced apoptosis (Figure 3B, WT). This effect of IFNγ required STAT1 because it was inhibited by 93% ($P<0.05$) in *Stat1*$^{-/-}$ macrophages (Figure 3B, *Stat1*$^{-/-}$). Thus, in atheromata, where macrophages are likely exposed to the combination of SRA ligands, ER stressors, and IFNγ, the role of STAT1 in macrophage apoptosis may be particularly important (below).

Cytosolic Calcium, TLR4, and CaMKII Activation Is Required for Stat1 Serine Phosphorylation and Apoptosis in FC-Loaded Macrophages

Three kinases that are able to catalyze serine phosphorylation of STAT1 are p38, extracellular signal-regulated kinase (ERK), and protein kinase C-δ.[23] However, using a combination of gene targeting and chemical inhibitors, we found that inhibiting these kinases did not abrogate FC-induced serine phosphorylation of STAT1 (data not shown). In the face of these negative data, we next asked whether 2 critical components of the multihit model, ER stress and TLR4 signaling, were necessary for STAT1 serine phosphorylation. The data in Figure 4A and 4B show that blocking FC-induced ER stress by the cholesterol trafficking inhibitor U18666A[4] or omitting thapsigargin from the fucoidan-plus-thapsigargin model markedly suppressed Ser-P-STAT1. In addition, FC-induced serine phosphorylation of STAT1 was almost completely prevented in TLR4-deficient macrophages (Figure 4C). Note that all of these manipulations also block macrophage apoptosis.[4,15]

Both ER stress and TLR4 signaling can affect cellular calcium metabolism (see Discussion).[12,13,15,26,27] Moreover, we recently showed that buffering cytosolic calcium with 1,2-bis[2-aminophenoxy]ethane-N,N,N',N'-tetraacetic acid tetrakis [acetoxymethyl ester] (BAPTA-AM) markedly inhibited both FC-induced and thapsigargin/fucoidan-induced apoptosis.[15] To test the role of cytosolic calcium in STAT1 serine phosphorylation, we incubated FC-loaded macrophages with increasing concentrations of BAPTA-AM or equivalent volumes of vehicle control. As shown in Figure 5A, BAPTA-AM suppressed FC-induced serine phosphorylation of STAT1 in a dose-dependent manner.

One mechanism by which cytosolic calcium might participate in STAT1 serine phosphorylation is by activating CaMKII, which may directly phosphorylate STAT1[28] and/or lead to its phosphorylation by enhancing TLR4 signaling (see Discussion).[15,29,30] As shown in Figure 5B, FC loading led to a rapid and marked enhancement of CaMKII threonine phosphorylation, which is a marker of its activation. At the 30- and 60-minute time points, the degree of activation was similar to that of the calcium ionophore A23187, a known potent activator of CaMKII. Similar results were found with fucoidan plus thapsigargin (data not shown). Note that the time course of CaMKII activation by FC loading or by thapsigargin plus fucoidan precedes the onset of STAT1 serine phosphorylation in these cells. To show a functional role for CaMKII activation in both STAT1 serine phosphorylation and apoptosis in FC-loaded macrophages, we used 2 structurally diverse CaMKII inhibitors. The data in Figure 5C and 5D show that the chemical CaMKII inhibitor KN93,[31] but not the inactive homologue KN92, and the peptide CaMKII inhibitor AIP[32] markedly suppressed FC-induced STAT1 serine phosphorylation. Most important, KN93 but not KN92 suppressed FC-induced apoptosis by 92% ($P<0.05$) (Figure 5E). Note that neither KN93 nor AIP decreased the uptake or ER trafficking of lipoprotein-derived FC or the induction of CHOP (data not shown and Figure 5D). In summary, these data indicate that cytosolic calcium and CaMKII are essential for STAT1 serine phosphorylation and apoptosis in the SRA-ER stress model. We also conducted experiments on 2 additional macrophage models, namely mouse bone marrow–derived and human peripheral blood–derived macrophages. In both of these cell types, the SRA–ER stress model exclusively induced STAT1 serine phosphorylation via a pathway mediated by cytosolic calcium and CaMKII (see the Figure in the online-only Data Supplement), suggesting the universality of this signaling pathway among macrophages.

STAT1 Is Serine Phosphorylated in Murine and Human Atherosclerotic Lesions, and STAT1 Plays a Role in Advanced Lesional Macrophage Apoptosis and Plaque Necrosis in Female *Ldlr*$^{-/-}$ Mice

To provide evidence for the relevance of Ser-P-STAT1 in atherosclerosis, we first used immunohistochemistry to

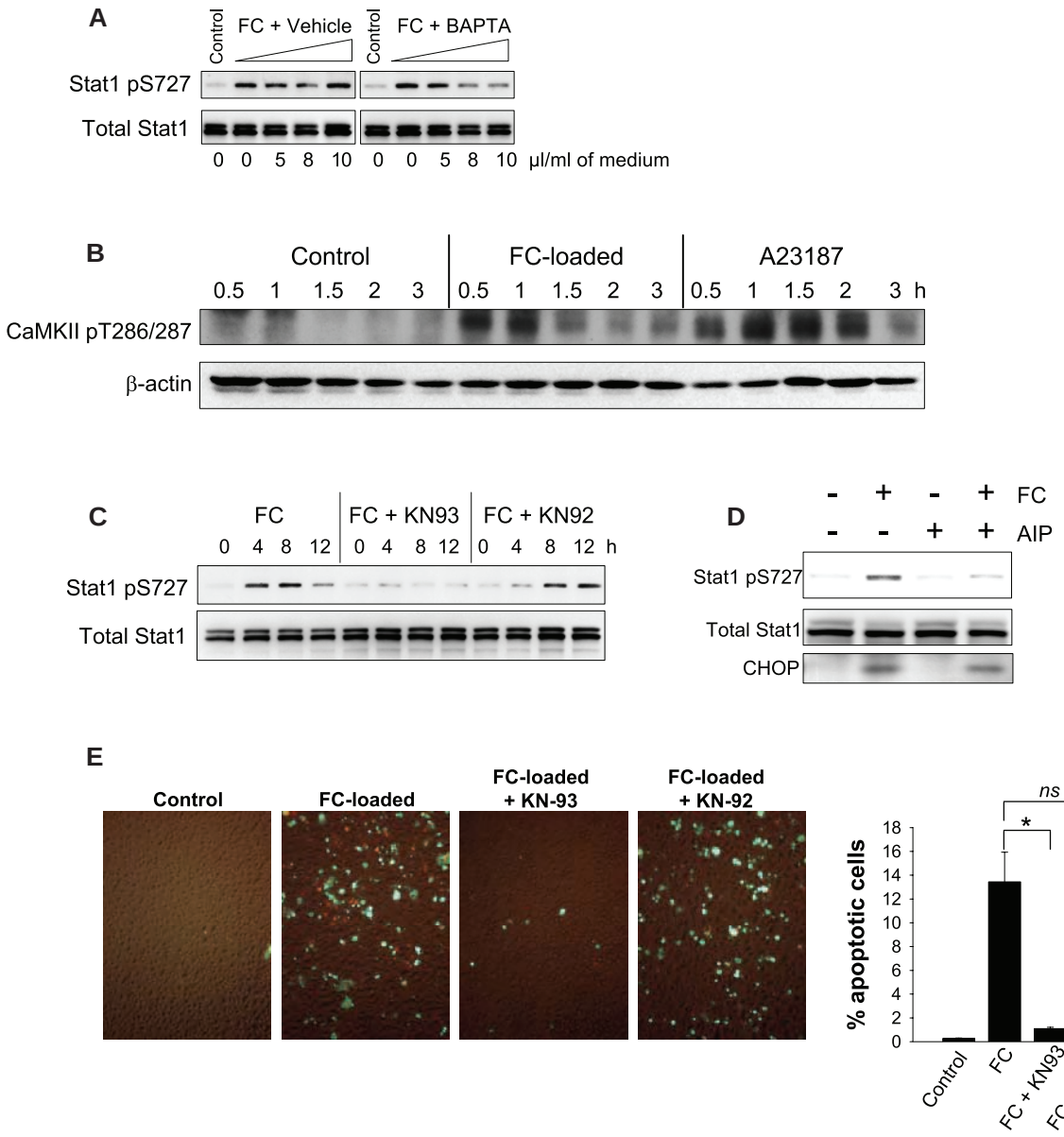


Figure 5. Cytosolic calcium and CaMKII activation is required for FC-induced STAT1 serine phosphorylation, and inhibition of CaMKII blocks FC-induced apoptosis. A through D, Whole-cell lysates were subjected to immunoblot analysis to detect STAT1 pS727, total STAT1, CaMKII pT286/287, β-actin, or CHOP, as indicated in the individual blots, under the following conditions. A, Macrophages were incubated for 8 hours in medium alone (Control); medium containing acetyl-LDL and 58035 plus vehicle control (FC+Vehicle); or medium containing acetyl-LDL, 58035, and increasing concentrations of BAPTA (FC+BAPTA). The indicated microliters of vehicle or BAPTA-AM stock solution (1 mg/mL) were added per 1 mL medium. B, Macrophages were incubated for the times indicated with medium alone (Control) or medium containing acetyl-LDL and 58035 (FC-loaded) or 2 μg/mL A23187. C, Macrophages were incubated for the times indicated with acetyl-LDL and 58035 (FC) or acetyl-LDL and 58035 plus either 10 μmol/L KN93 or 10 μmol/L KN92 (FC+KN93 or FC+KN92). D, Macrophages were incubated for 8 hours with acetyl-LDL and 58035 (FC), 10 μmol/L AIP, or acetyl-LDL, 58035, and AIP. E, Macrophages were incubated for 24 hours in medium alone (Control) or medium containing acetyl-LDL and 58035 (FC-loaded) or acetyl-LDL and 58035 plus either 10 μmol/L KN93 or 10 μmol/L KN92 (FC-loaded+KN93 or FC-loaded+KN92). Apoptosis was assayed and quantified as in Figure 1. For all experiments involving KN93, KN92, or AIP, the macrophages were pretreated for 1 hour with medium alone or medium containing these inhibitors before FC loading. *$P<0.01$ by Bonferroni after ANOVA.

assess expression of Ser-P-STAT1 in murine and human atheromata (Figures 6 and 7). In mouse lesions, Ser-P-STAT1 was present in numerous macrophage foam cells, as assessed by staining adjacent sections with anti–Mac-3 antibody (Figure 6A and 6B) and Oil Red O (Figure 6D). As illustrated by these images, Ser-P-STAT1 staining also was observed in the endothelial cells lining the lumen, which was PECAM-1 positive (not shown), and in smooth muscle cells in the media, which were α-actin positive (Figure 6F). In human lesions, staining of Ser-P-STAT1 was found in the advanced stages called pathological intimal thickening and fibroatheroma (Figure 7B and 7C)

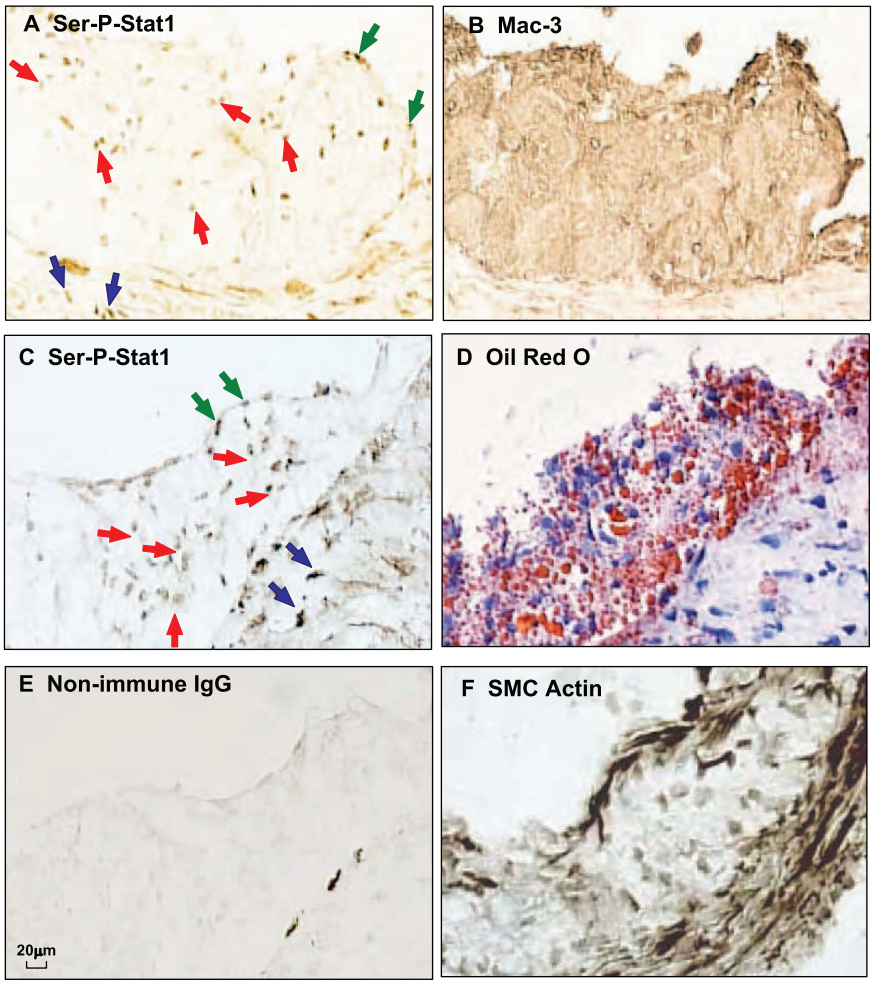


Figure 6. STAT1 is serine phosphorylated in atherosclerotic lesions from *Ldlr*$^{-/-}$ mice. Adjacent frozen sections of an aortic root lesion from an *Ldlr*$^{-/-}$ mice fed a Western-type diet for 12 weeks were immunostained with anti–Ser-P-STAT1 or anti-Mac3 (macrophages) (A and B) or anti-Ser-P-STAT1, oil red O, nonimmune immunoglobulin G (IgG), and α-actin (C through F). Note examples of brown stain in the nuclei of the intimal cells (red arrows), endothelial cells (green arrows), and smooth muscle cells (SMC) in the media (blue arrows). The dark streaks at the intima-media interface in E represent nonspecific staining.

but not in the early stage of diffuse intimal thickening (Figure 7A). In the advanced lesions, most of the Ser-P-STAT1 colocalized with macrophages (Figure 7B and 7C). Note that Ser-P-STAT1 was found in the nuclei of these cells (Figure 7C, bottom middle) and in areas that were TUNEL positive, a marker of apoptosis (Figure 7C, bottom right). Of interest, some of the Ser-P-STAT1 in the most advanced fibroatheroma was found in macrophages surrounding necrotic areas (Figure 7C, top middle, asterisk).

To further investigate a causal link between STAT1 and lesional macrophage apoptosis, we compared advanced plaques of Western diet–fed *Ldlr*$^{-/-}$ mice reconstituted with either WT or *Stat1*$^{-/-}$ bone marrow. The mice were fed the Western diet for 10 or 12 weeks. Plasma lipoprotein cholesterol and body weight were similar between the 2 groups of mice (Figure 8A for 10-week protocol; data not shown for 12-week protocol). In the 10-week study, overall lesion areas were similar (Figure 8B and 8C). However, the number of TUNEL-positive cells in macrophage-rich regions was decreased by 61% ($P=0.034$) in the *Stat1*$^{-/-}$→*Ldlr*$^{-/-}$ lesions, and a trend toward decreased plaque necrosis existed that did not quite reach statistical significance ($P=0.078$) (Figure 8C). Note that total macrophage area was not affected by STAT1 deficiency ($120.0\pm11.8\times10^3$ and $111.5\pm21.3\times10^3$ μm^2 in WT and *Stat1*$^{-/-}$ bone marrow recipients, respectively; $P=0.72$; see Discussion).

Plaque necrosis likely results from the eventual cellular necrosis of macrophages that become apoptotic but are not subsequently cleared by phagocytes.[1,2] Therefore, we predicted that as the lesions in the 2 groups of mice progressed, the difference in necrotic core areas would become statistically significant, whereas apoptotic macrophages per se would become less numerous and less different between the 2 groups of mice. As shown by the data in Figure 8D, the necrotic cores were larger in the 12-week-diet mice, and a statistically significant difference was present in the necrotic core area (34% decrease in the *Stat1*$^{-/-}$→*Ldlr*$^{-/-}$ lesions; $P=0.02$) but not the number of TUNEL-positive cells. In summary, STAT1 deficiency in bone marrow–derived cells in *Ldlr*$^{-/-}$ mice has a substantial protective effect on apoptosis in the macrophage-rich lesions of advanced plaques and on plaque necrosis.

Discussion

Increasing evidence from a number of laboratories suggests that an ER stress–based model of macrophage apoptosis plays an important role in advanced lesional macrophage death and plaque necrosis.[4–9] The work reported here adds critical new components to this model by demonstrating essential roles for STAT1 and CaMKII in macrophage apoptosis in vitro and for STAT1 in advanced lesional macrophage apoptosis and plaque necrosis in vivo.

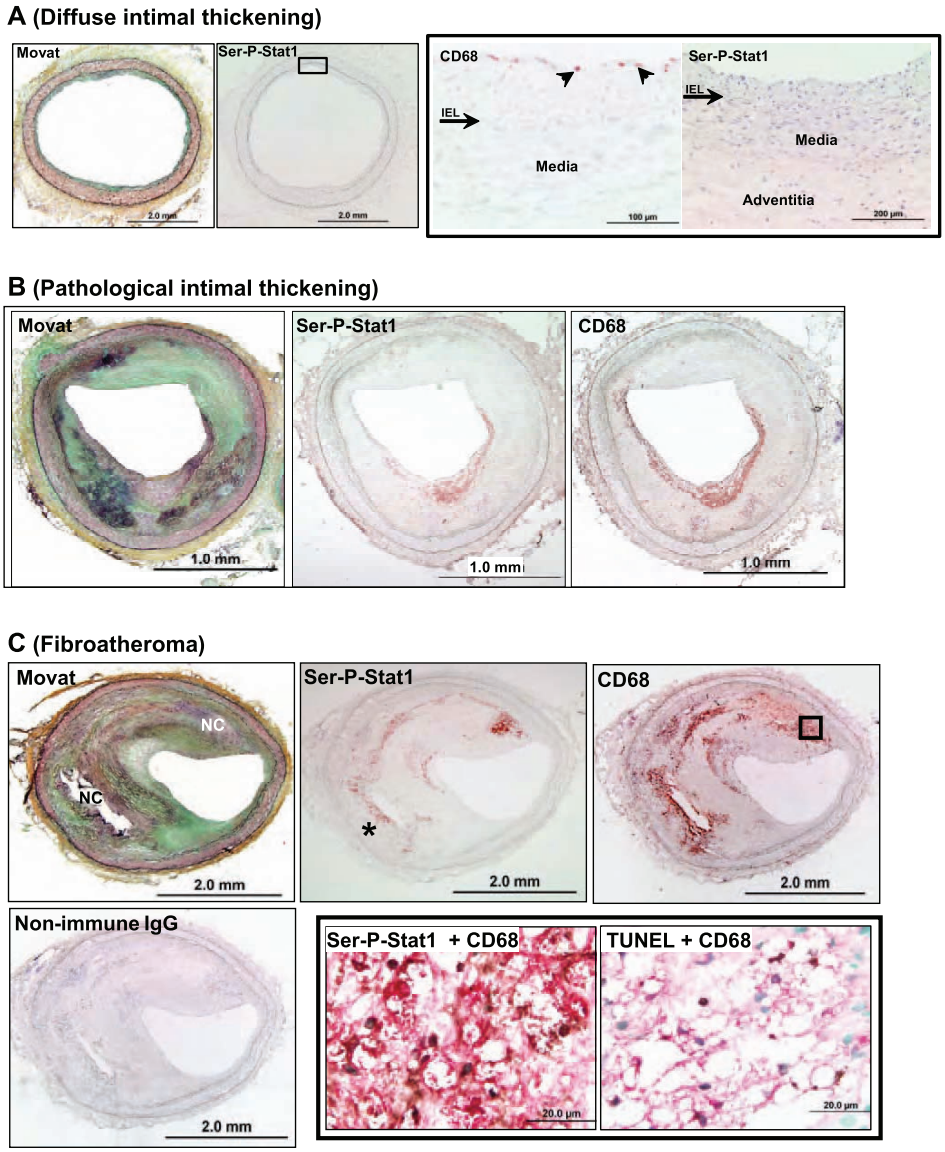


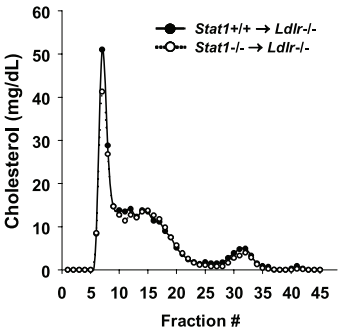
Figure 7. Ser-P-STAT1 is present in advanced human coronary atheromata but not in diffuse intimal thickening. The sections were stained with Movat pentachrome, anti–Ser-P-STAT1, anti-CD68, and nonimmune immunoglobulin (IgG) as indicated. A, Diffuse intimal thickening. The CD68 and Ser-P-STAT1 images on the right are higher magnifications of the area indicated by the box in the low-magnification Ser-P-STAT1 image. As shown in the higher-magnification images, only a few CD68-positive macrophages are present directly under the endothelium (arrowheads). Ser-P-STAT1 was not detected. Arrow indicates internal elastic lamina (IEL). B, Pathological intimal thickening. Ser-P-STAT1 staining coincides with CD68-positive macrophages. C, Fibroatheroma. Ser-P-STAT1 staining coincides with CD68-positive macrophages. Some of the Ser-P-STAT1 staining is in macrophages surrounding a necrotic area (asterisk). The lower middle and right images are higher magnifications of the area indicated by the box in the low-magnification CD68 image. The lower middle image shows the result of double immunostaining with anti–Ser-P-STAT1 (dark punctate structures) and anti-CD68 (red), demonstrating Ser-P-STAT1 in the nuclei of macrophages. The lower right image shows the result of double immunostaining with TUNEL (dark, punctate structures) and anti-CD68 (red), demonstrating apoptotic macrophages. Nuclei of nonapoptotic cells are stained green. Note that exact alignment of the nuclei is not possible because the sections are from separate tissue slices.

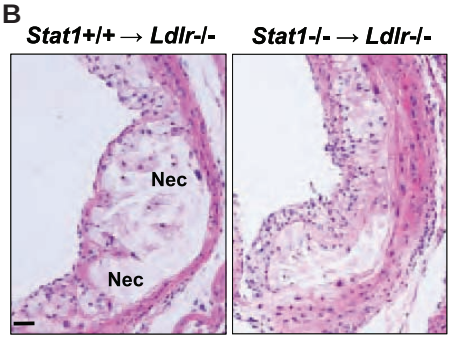
Further studies are required to define at a precise molecular level how the proapoptotic components elucidated in this study fit into the overall scheme of the multihit model of macrophage apoptosis. Our working hypothesis is depicted in Figure 9. We suggest that ER stress triggers 2 key proapoptotic processes: UPR/CHOP and another pathway in which ER stress–induced cytosolic calcium activates CaMKII, which in turn leads to serine phosphorylation of proapoptotic STAT1. Activation of the TLR4-MyD88 pathway by SRA ligands, which is critical for apoptosis,[15] also contributes to STAT1 serine phosphorylation. SRA ligands additionally promote apoptosis through SRA-dependent suppression of prosurvival IFNβ.[14,15]

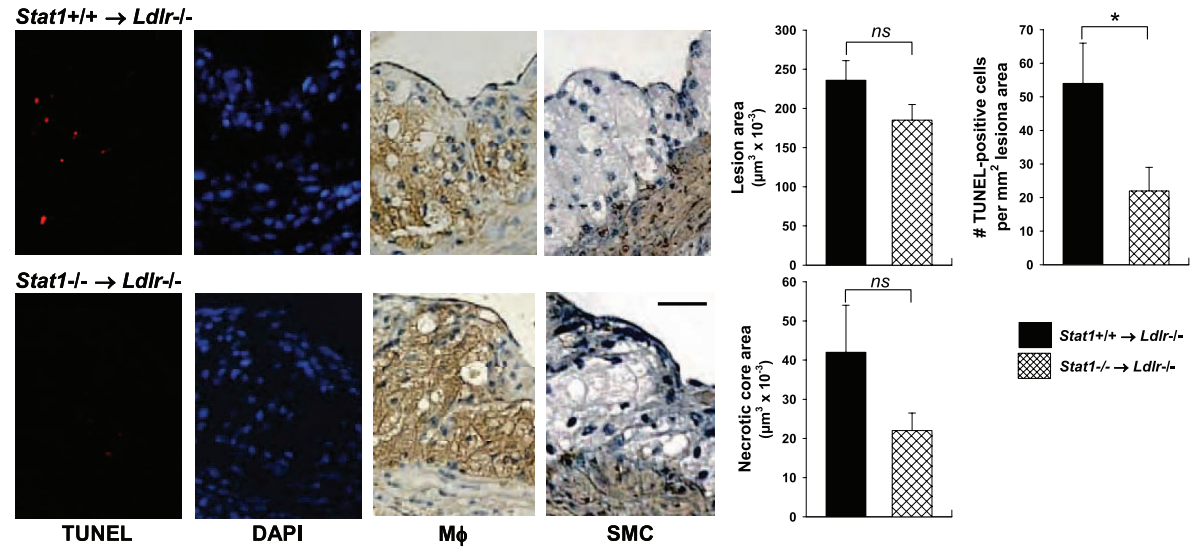
This scheme raises a number of critical issues that require further investigation. Among these is whether STAT1 serine phosphorylation per se is required for apoptosis, which is consistent

A

	Stat1+/+ → Ldlr-/- (n = 13)	Stat1-/- → Ldlr-/- (n = 9)
T. Chol (mg/dl)	636.5 ± 59.8	597.8 ± 63.8
HDL-Chol (mg/dl)	55.6 ± 3.2	56.1 ± 4.0
Body wt. (g)	20.0 ± 0.6	20.0 ± 0.8







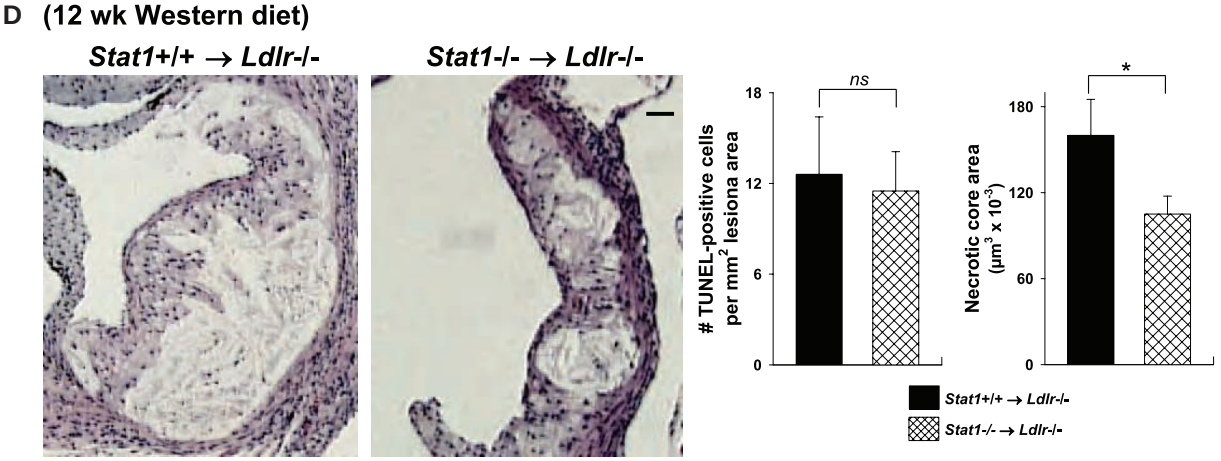


Figure 8. STAT1 plays a role in advanced lesional macrophage apoptosis and plaque necrosis in female *Stat1*$^{-/-}$→*Ldlr*$^{-/-}$ mice. A, The table shows plasma cholesterol and body weight of *Ldlr*$^{-/-}$ mice transplanted with *Stat1*$^{+/+}$ or *Stat1*$^{-/-}$ bone marrow and then fed a Western-type diet for 10 weeks starting 6 weeks after transplantation. The graph shows pooled plasma samples from 3 *Stat1*$^{+/+}$ and 3 *Stat1*$^{-/-}$ recipient mice that were fractionated by fast protein liquid gel-filtration chromatography and then assayed for cholesterol. None of the differences in cholesterol, lipoproteins, or body weight were statistically significant. T. Chol indicates total cholesterol; HDL, high-density lipoprotein. B, Hematoxylin and eosin staining of proximal aortas from *Ldlr*$^{-/-}$ mice fed a Western diet for 10 weeks that were transplanted with bone marrow from *Stat1*$^{+/+}$ and *Stat1*$^{-/-}$ mice. Total lesion area was 493.7±40.5 and 380.6±24.4 μm^2 in *Stat1*$^{+/+}$→*Ldlr*$^{-/-}$ and *Stat1*$^{-/-}$→*Ldlr*$^{-/-}$ mice, respectively. Bar=20 μm. C, TUNEL (red), DAPI (blue), macrophage (brown), and SMC (brown) staining of lesions similar to those in B. Bar=20 μm. The graph shows quantification of lesion area, TUNEL-positive cells, and necrotic area (Nec) in the lesions of *Stat1*$^{+/+}$→*Ldlr*$^{-/-}$ and *Stat1*$^{-/-}$→*Ldlr*$^{-/-}$ mice. *P=0.034 by Student t test. D, Hematoxylin and eosin staining of proximal aortas from *Ldlr*$^{-/-}$ mice fed a Western diet for 12 weeks that were transplanted with bone marrow from *Stat1*$^{+/+}$ and *Stat1*$^{-/-}$ mice (n=18 for both groups of mice). Bar=20 μm. The graph shows quantification of TUNEL-positive cells and necrotic area in the lesions of *Stat1*$^{+/+}$→*Ldlr*$^{-/-}$ and *Stat1*$^{-/-}$→*Ldlr*$^{-/-}$ mice. P=0.02 by Student t test.

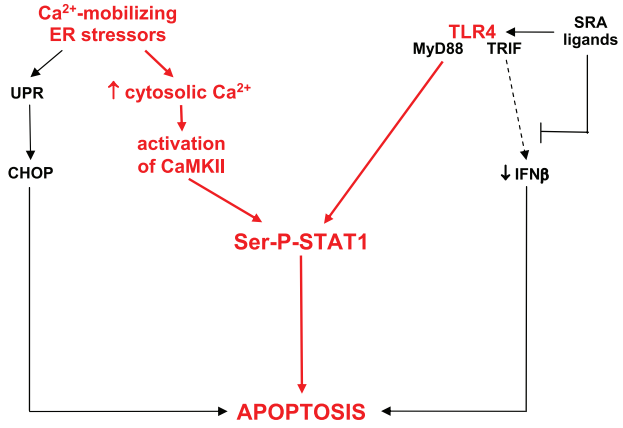


Figure 9. Integration of calcium, CaMKII, and STAT1 into the multihit pathway of macrophage apoptosis. According to this working hypothesis, ER stress–induced increase in cytosolic calcium triggers 2 proapoptotic hits: UPR/CHOP and a pathway involving CaMKII and Ser-P-STAT1. TLR4 activation also contributes to STAT1 serine phosphorylation. Ser-P-STAT1 is depicted as a separate pathway from CHOP because studies with *Chop*$^{-/-}$ and *Stat1*$^{-/-}$ macrophages showed that CHOP is neither upstream nor downstream of Ser-P-STAT1 (data not shown). See Discussion for details and for a description of the areas of uncertainty in this model.

with our data and with previous work showing a proapoptotic role of Ser-P-STAT1 in apoptosis in other systems.[33] However, definitive proof requires comparing SRA/ER stress–induced apoptosis in macrophages containing S727- with Y701-mutated STAT1.[22,33] Until then, we cannot definitively rule out the possibility that apoptosis requires Y701 phosphorylation and that Tyr-P-STAT1 in our SRA–ER stress model is below the limit of immunoblot detection. In pilot studies, we found that apoptosis induced by thapsigargin and fucoidan was markedly suppressed in peritoneal macrophages from S727A-STAT1 knock-in mice, but results with FC-induced apoptosis were difficult to interpret because of an as-yet-undefined compensatory pathway (J.M.T., W.L., T. Decker, PhD, and I.T., unpublished data, 2007). Assuming that Ser-P-STAT1 is a key apoptosis mediator in this pathway, the next goal is to elucidate whether and how serine-only phosphorylated STAT1 affects gene transcription in a manner that promotes apoptosis.[33–35] A related issue is the precise mechanism by which STAT1 is serine phosphorylated and how this process is linked to both CaMKII and TLR4 (Figure 9). One obvious possibility is that CaMKII directly phosphorylates STAT1, as has been described previously in other models.[28] If this is the case in our model, a link to TLR4 could occur through IP3 receptor–mediated elevation of cytosolic calcium,[26] leading to a further increase in CaMKII activation. Alternatively, TLR4 signaling may stimulate STAT1 serine phosphorylation more directly, as has been reported in other models using the TLR4 ligand lipopolysaccharide.[29,30]

The impetus for this study was to explore pathways that may be involved in promoting macrophage apoptosis in atherosclerosis. The ultimate significance of lesional macrophage apoptosis likely depends on lesion stage.[1,2] In early lesions, rapid and efficient phagocytic clearance of apoptotic macrophages appears to limit lesion cellularity and progression. Of interest, STAT1 may have a separate role in these early lesions that is independent of macrophage death because STAT1 deficiency in *Apoe*$^{-/-}$ mice blocks foam cell formation and early lesion development.[36] In advanced lesions, however, evidence exists that clearance of apoptotic cells is defective, leading to postapoptotic macrophage necrosis, inflammation, and eventually overall plaque necrosis.[1,2] In this context, the multihit model of macrophage apoptosis is likely most relevant to advanced lesions. For example, immunoblots have shown that CHOP is expressed only in advanced lesions[7] and that manipulation of ER stress in vivo is positively associated with advanced lesional necrosis, not negatively associated with early lesion progression.[5,37] In the case of STAT1 deficiency, a clear trend toward decreased plaque necrosis was present. However, the maximum effect on plaque necrosis may lag behind that of macrophage apoptosis because plaque necrosis likely results from the progressive coalescence of apoptotic macrophages after they become secondarily necrotic.[1,2] Another prediction from this idea and from the fact that the anti-macrophage antibody used in our study recognizes prenecrotic apoptotic macrophages is that total macrophage area should be similar in *Stat1*$^{+/+}$→*Ldlr*$^{-/-}$ and *Stat1*$^{-/-}$→*Ldlr*$^{-/-}$ lesions, exactly as we observed experimentally. More fundamentally, we clearly did not observe an increase in lesion area in the *Stat1*$^{-/-}$ group, which is what is found when early lesional macrophage apoptosis is blocked.[38] In terms of other studies linking STAT1 to advanced plaque progression, in vivo data suggest that interleukin-10, which suppresses STAT1 activity,[39] may protect advanced atheromata from macrophage apoptosis and plaque necrosis.[40,41] Moreover, Koga et al[25] reported that blocking the function of the STAT1 activator IFNγ stabilized advanced plaques in *Apoe*$^{-/-}$ mice. Thus, pending further in vivo studies, local inhibition of STAT1 activity may represent a potentially promising therapeutic strategy to prevent the progression of relatively benign lesions to those with increased macrophage apoptosis and plaque necrosis.

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Disclosures

None.

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CLINICAL PERSPECTIVE

In industrialized societies, virtually all young adults have atherosclerosis. Most of these lesions are asymptomatic and will remain so for the rest of the person's life. However, a small percentage will progress to a dangerous stage involving plaque breakdown, acute luminal thrombosis, and acute vascular events like myocardial infarction and sudden cardiac death. Thus, a major goal is to elucidate the cellular-molecular events involved in benign-to-vulnerable plaque progression. A key feature of vulnerable plaques is necrotic cores, which likely promote plaque breakdown and acute thrombosis. Necrotic cores are "graveyards of dead macrophages," a prominent cell type in atherosclerosis. This study used a cell-culture model of macrophage death to explore death-promoting molecules that may be relevant to advanced atherosclerosis. These experiments revealed an important role for a calcium-signaling pathway involving 2 molecules, calcium/calmodulin-dependent protein kinase II and signal transducer and activator of transcription-1 (STAT-1). Both mouse and human advanced atheromata have activated STAT-1. Most important, when macrophages were made deficient in STAT-1 in a mouse model of advanced atherosclerosis, macrophage death and plaque necrosis were diminished. Two important caveats of this study need to be mentioned. First, the processes of macrophage death and plaque necrosis are complex, so the calcium/calmodulin-dependent protein kinase II–STAT1 pathway represents only 1 piece of the puzzle. Second, the mouse is a poor model of plaque disruption and acute thrombosis. Thus, additional studies are needed to explore other pathways involved in advanced lesional macrophage death, and improved mouse models are required to prove the hypothesis that macrophage death and plaque necrosis promote plaque disruption and acute thrombosis. Nonetheless, this study provides important new molecular-cellular information related to the progression of advanced atherosclerotic lesions—information that someday may be translated into therapy designed to block benign-to-vulnerable plaque transformation.

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Extracellular Nampt Promotes Macrophage Survival Via a Non-Enzymatic Interleukin-6/STAT3 Signaling Mechanism*

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Macrophages play key roles in obesity-associated pathophysiology, including inflammation, atherosclerosis, and cancer, and processes that affect the survival-death balance of macrophages may have an important impact on obesity-related diseases. Adipocytes and other cells secrete a protein called extracellular nicotinamide phosphoribosyltransferase (eNampt; aka PBEF, visfatin), and plasma levels of eNampt increase in obesity. Herein we tested the hypothesis that eNampt could promote cell survival in macrophages subjected to endoplasmic reticulum (ER) stress, a process associated with obesity and obesity-associated diseases. We show that eNampt potently blocks macrophage apoptosis induced by a number of ER stressors. The mechanism involves a two-step sequential process: rapid induction of interleukin 6 (IL-6) secretion, followed by IL-6-mediated autocrine/paracrine activation of the pro-survival signal transducer STAT3. The ability of eNampt to trigger this IL-6-/STAT3 cell-survival pathway did not depend on the presence of the Nampt enzymatic substrate nicotinamide in the medium; could not be mimicked by the Nampt enzymatic product nicotinamide mononucleotide (NMN); was not blocked by the Nampt enzyme inhibitor FK866; and showed no correlation with enzyme activity in a series of site-directed mutant Nampt proteins. Thus, eNampt protects macrophages from ER stress-induced apoptosis by activating an IL-6/STAT3 signaling pathway via a non-enzymatic mechanism. These data suggest a novel action and mechanism of eNampt that could affect the balance of macrophage survival and death in the setting of obesity, which in turn could play important roles in obesity-associated diseases.

Macrophage biology is intimately related to obesity-associated pathology. For example, inflammatory macrophages are numerous in adipose tissue of obese individuals (1, 2), and macrophage-mediated inflammation is a critical component of obesity-induced insulin resistance (2, 3). Macrophages also play a critical role in the progression of atherosclerosis, which is the process behind the leading cause of death in obese and insulin-resistant subjects, atherothrombotic vascular disease (4). Obesity is associated with an increased risk for certain cancers, and tumor-associated macrophages (TAMs) play a critical role in the development of these tumors (5-7). In all processes involving macrophages, a critical determinant of outcome is the survival-death balance of the cells. For example, macrophage

apoptosis, when coupled with effective phagocytic clearance (efferocytosis) of the dead cells, suppresses inflammation (8). In atherosclerosis, macrophage apoptosis limits plaque progression in relatively early lesions, where efferocytosis is intact (9-12). In cancer, targeted apoptosis of TAMs suppresses tumor development (7). Thus, increased macrophage survival in obesity, perhaps mediated by circulating bioactive proteins whose levels change with increased body mass index ("adipocytokines") (13, 14), may promote the aforementioned obesity-associated diseases.

An adipocytokine that has received much recent attention is a secreted form of an enzyme called nicotinamide phosphoribosyltransferase (Nampt; aka PBEF, visfatin), which converts nicotinamide to nicotinamide mononucleotide (NMN) (15, 16). In vertebrates, intracellular Nampt (iNampt) plays an essential role in the biosynthesis of NAD from nicotinamide (17). However, adipocytes and other cells secrete Nampt through a non-classical, brefeldin A-independent pathway into the extracellular medium *in vitro* and into the circulation *in vivo* (18-20). Recent studies have shown that plasma levels of circulating Nampt/PBEF/visfatin, referred to here as extracellular Nampt (eNampt), are elevated in obese humans, including those with type 2 diabetes (21-24).

eNampt has been implicated in a number of biological processes. For example, eNampt was initially identified as a cytokine, called pre-B cell colony enhancing factor (PBEF), that can promote the maturation of B cell precursors and inhibit neutrophil apoptosis (19). Later work revealed pro-inflammatory actions of the protein *in vitro* and in mice, as well as a positive association among obesity, eNampt plasma levels, and inflammation in humans (14, 25). The issue as to whether these cytokine-like effects of eNampt depend on Nampt enzymatic activity has generally not been investigated, although a recent study has implicated Nampt enzymatic activity in enhancing the inflammatory response of macrophages to LPS (26).

In another set of studies in which eNampt was named "visfatin," a group of investigators reported that the protein could activate insulin receptor signaling in cultured cells and *in vivo* through direct interaction with the insulin receptor (27). However, the authors have recently retracted the publication due to problems reproducing these results (28). More recently, Revollo *et al*. (20) showed that eNampt enhances glucose-stimulated insulin secretion in pancreatic β cells *in vitro* and in non-obese mice. This effect of the protein was shown not to be mediated through activation of insulin receptor signaling but rather involved the extracellular enzymatic conversion of nicotinamide to NMN by eNampt, leading to increased NAD biosynthesis in β cells (20).

Given eNampt levels are increased in obesity and that macrophages play important roles in obesity-associated diseases, we questioned at a cell biological level whether eNampt might influence the survival-death balance of macrophages. In particular, increased macrophage survival might enhance the proposed inflammatory effects of eNampt (above). We focused on an apoptosis inducer—endoplasmic reticulum (ER) stress—that occurs in obesity and is likely to be relevant to obesity-associated diseases. For example, ER

stress is a critical process in obesity-associated insulin resistance and inflammation (29, 30), and evidence suggests that ER stress-induced macrophage apoptosis occurs in atherosclerotic lesions (31-35), particularly in the setting of insulin resistance (36). ER stress is also prominent in tumors, where it may affect TAMs (37). Using models of ER stress-induced apoptosis, we show here that eNampt potently blocks macrophage apoptosis. The cell survival pathway is mediated by STAT3, which is activated in an autocrine/paracrine manner by eNampt-induced IL-6. The ability of eNampt to activate STAT3 involves neither insulin receptor signaling nor the nicotinamide phosphoribosyltransferase activity of the protein, suggesting a non-enzymatic signaling role for the protein in this pathway. These findings have potential implications for how obesity might affect macrophage apoptosis-survival balance, with potential implications for macrophage-mediated obesity-associated diseases, as well as for the mechanisms of action of eNampt.

EXPERIMENTAL PROCEDURES

Materials—Cell culture media and reagents were from Invitrogen Life Technologies, Inc. Chemical and biochemical reagents were from Sigma unless specified below. The acyl-CoA:cholesterol acyl transferase (ACAT) inhibitor 58035 (3-[decyl dimethyl silyl]-*N*-[2-(4-methyl phenyl)-1-phenethyl]propanamide) (SA 58-035; cat #S9318) and 7-ketocholesterol were purchased from Sigma. A stock solution of 58035 was prepared at a concentration of 10 mg/ml in dimethyl sulfoxide and stored at –20^{o}C. Recombinant murine eNampt (His-tagged) was from Axxor, LLC (San Diego, CA). Low-density lipoprotein (LDL; *d* 1.020-1.063 g/ml) was isolated from fresh human plasma by preparative ultracentrifugation as previously described (38). Acetyl-LDL was prepared by reaction of LDL with acetic anhydride (39). Recombinant murine IL-6 and IL-10; neutralizing antibodies against IL-6, IL-6R, IL-10, and IL-10R; and control IgG were from R&D Systems. JAK1/2 inhibitor AG490 was from EMD. Antibodies against STAT3 and phospho-STAT3 were from Cell Signaling Technology. Antibodies against Mcl-1 (rabbit polyclonal), Bcl-xL (mouse monoclonal), Bcl-2 (rabbit polyclonal), IAP (goat polyclonal), and β-actin were from Santa Cruz Biotechnologies, Inc. AIM/CD5L (rabbit polyclonal) was from AbD Serotec (Oxford, UK). Scrambled and Stat3 siRNA species were from Qiagen (Valencia, CA). siRNA #1 (SI01435287) and #2 (SI01435301) were designed from regions situated between base pairs 1050-1100 and 100-150, respectively, of Stat3 mRNA; the exact siRNA sequences are proprietary. Scrambled control siRNA (AllStars® Negative Control siRNA) from Qiagen was designed to have no homology to any known mammalian gene (https://www1.qiagen.com/Products/GeneSilencing/AllStarRNAiControls/AllStarsNegativeControls.aspx). FK866, a non-competitive allosteric inhibitor of Nampt, was a kind gift of Dr. L. Tong, Biological Sciences, Columbia University, New York, NY.

Preparation of Mouse Peritoneal Macrophages and Bone-Marrow-Derived Macrophages—Unless otherwise noted, the mice used in this study were female C57BL6/J mice, 8 to 10 weeks of age. Peritoneal macrophages were elicited by intraperitoneal (i.p.) injection of methyl-BSA

(mBSA) in mice previously immunized with this antigen as previously described (40). The cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin/streptomycin, and 20% L-cell-conditioned medium for 24-48 h, at which time the cells were ~80-90% confluent. Mouse bone marrow cells were collected by flushing femurs with DMEM. The suspension was centrifuged at 1000 *x g* for 5 min, and the cell pellet was resuspended in macrophage medium (above) and cultured for 9-11 days to allow differentiation into mature macrophages.

Apoptosis Induction and Assays—Apoptosis was induced by simultaneous or separate activation of the unfolded protein response (UPR) and pattern recognition receptors (PRRs) (31, 32, 41, 42) or, in the case of Fig. 1B, with 5 μg/ml staurosporine. For simultaneous UPR/PRR activation, macrophages were incubated for 20 h with 50 μg/ml acetyl-LDL plus 10 μg/ml of the ACAT inhibitor, SA 58-035. For separate UPR/PRR activation, macrophages were incubated for 24 h with 5 μg/ml tunicamycin plus 25 μg/ml fucoidan. At the end of the incubation period, macrophages were assayed for externalization of phosphatidylserine, a marker of early-to-mid-stage apoptosis, by staining with Alexa-488-labelled annexin V. Late-stage apoptosis, as indicated by increased membrane permeability, was assayed by staining with propidium iodide (PI) (43). For Figs. 3 and 4F, the annexin data were verified by TUNEL (Tdt-mediated dUTP nick end labeling) analysis, using the *in-situ* cell death detection kit, TMR red, from Roche (see text). Cells were viewed immediately with an Olympus IX-70 inverted fluorescence microscope, and four representative fields (approximately 1,000 cells) for each condition were counted. The data are expressed as percentage of apoptotic cells per total cells.

Immunoblotting—Whole-cell lysates were prepared by lysing cells in RIPA buffer ((25 mM Tris, pH 7.4, 150 mM KCl, 5 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) at the end of treatments. The lysates were electrophoresed on 4–20% gradient SDS–PAGE gels and transferred to 0.22-μm nitrocellulose membranes. The membrane was blocked for 1 h at room temperature in Tris-buffered saline, 0.1% Tween-20 (TBST) containing 5% (w/v) nonfat milk and then incubated for 18 h with the primary antibody in TBST containing 5% (w/v) nonfat milk or 5% BSA at 4^{o}C. The blots were then incubated with the appropriate secondary antibody coupled to horseradish peroxidase, and proteins were detected by ECL chemiluminescence (Pierce).

Stat3 siRNA Experiments—Scrambled and Stat3 siRNA (50 pg/well) were transfected into 50% confluent monolayers of peritoneal macrophages using Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturers instructions. After 4 h of transfection, the media were replaced, and 60 h later the indicated experiments were conducted.

In Vivo Administration of eNampt—Male C57BL/6J mice were injected *i.p.* with 1 ml 3% thioglycollate to elicit peritoneal macrophages. Four days later, the mice were injected *i.v.* with 5 μg eNampt in 0.5 ml PBS or with PBS alone as control. Peritoneal macrophages were harvested 12 h after the eNampt injection and then immediately assayed for phospho-STAT3 by immunoblotting.

Mutant Nampt Proteins and Kinetic Analysis—His-tagged wild-type and mutant Nampt proteins were expressed as previously described (44). The proteins were purified to at least 95% pure using one-step Histrap purification. The nicotinamide phosphoribosyltransferase activity of Nampt was measured as previously described (16, 20, 44). For kinetics studies on His-tagged wild-type and mutant Nampt proteins, each reaction consists of 100 µl of 20 mM Tris, pH 8.0, 50 mM NaCl, 2 mM DTT, and 12.5 mM $MgCl_2$, 2.5 mM ATP, 0.03% BSA, 1.5% ethanol, 13 µg/ml His-tagged Nmnat, 100 µg/ml alcohol dehydrogenase, 500 µM phosphoribosyl pyrophosphate, and varying concentrations of nicotinamide. Depending on different catalytic abilities, different amounts of His-Nampt were used for the wild-type and the mutants. Catalytic constants K_m and k_{cat} were determined for His-tagged mutant and wild-type proteins by analyzing plots of initial rate measurements at 25°C under varying nicotinamide concentrations using the KaleidaGraph 3.6 program (Synergy Software), which calculates constants K_m and k_{cat} automatically.

Statistics—Data are presented as mean ± S.E.M. Unless otherwise noted, the number of samples for each group (n) = 4. Absent error bars in the bar graphs signify S.E.M. values smaller than the graphic symbols. ANOVA followed by Tukey post-test (GraphPad Prism 4 version 4.03) was used to determine statistical significance among all groups.

RESULTS

Extracellular Nampt Suppresses ER-Stress-Induced Macrophage Apoptosis Via Activation of STAT3—ER stress-induced macrophage apoptosis requires activation of the UPR-CHOP pathway plus a second "hit" involving pattern recognition receptor (PRR) activation (32, 42). In one example of this model that may be relevant to atherosclerosis, macrophages are incubated with modified lipoproteins that carry out both hits: the lipoproteins bind a PRR called the type A scavenger receptor; and, in the absence of cholesterol re-esterification, lipoprotein-derived unesterified cholesterol accumulates in the ER, alters the ER membrane, and triggers the UPR-CHOP pathway (31, 32, 41). Using this model, we found that pre-incubation of the macrophages with recombinant eNampt markedly suppressed apoptosis (**Fig. 1A**).

There is evidence that the oxidized sterol 7-ketocholesterol is present in atherosclerotic lesions, and 7-ketocholesterol activates the UPR and is pro-apoptotic (35, 45). As shown in the *left graph* in **Fig. 1B**, 7-ketocholesterol induced apoptosis in macrophages, and the apoptotic response was markedly suppressed by eNampt. A more broadly applicable, sterol-independent example of this apoptosis model involves incubating macrophages with the combination of a low-dose UPR activator (tunicamycin) and a PRR ligand (fucoidan) (32). eNampt was also very effective at blocking apoptosis in this model (*right graph* in **Fig. 1B**). Interestingly, eNampt also had some survival effect in a non-UPR based model of apoptosis, incubation with the phosphatase inhibitor staurosporine. Although the decrease in staurosporine-induced apoptosis was statistically significant ($P<0.01$), the effect was considerably less than that observed with UPR-induced apoptosis (**Fig. 1B**).

To pursue mechanism, we first examined the effect of eNampt on PRR expression, which is essential for ER stress-induced apoptosis (32). We found that treatment of macrophages with eNampt had no effect on the expression of the type A scavenger receptor or another PRR, CD36, both of which serve as second hits in UPR-induced apoptosis (Refs. (32, 42) and data not shown). Next, in view of the previously postulated, and since retracted, role of eNampt (visfatin) as an insulin receptor agonist (27, 28), we determined whether eNampt could block apoptosis in macrophages harvested from insulin receptor-null mice (36). We found that thapsigargin/fucoidan-induced apoptosis was blocked by eNampt to the same degree in these macrophages as in wild-type macrophages (data not shown).

We next considered another possible mechanism for the cell-survival effect of eNampt, namely, activation of one or more cell-survival signaling pathways. Activated STAT3 can act as a survival signal in a number of cell types (46). As shown in **Fig. 2A**, eNampt is a potent inducer of STAT3 tyrosine phosphorylation, a marker of STAT3 activation, and the effect was seen with an eNampt concentration as low as 50 ng/ml (**Fig. 2B**). The ability of eNampt to activate STAT3 was not dependent on macrophage insulin signaling, because the response was the same in insulin receptor-null vs. wild-type macrophages (data not shown). However, STAT3 activation was inhibited by the JAK kinase inhibitor AG490 (tyrphostin) (**Fig. 2C**), consistent with the role of JAK kinases in activation of STAT3 (47). Importantly, STAT3 activation occurred *in vivo*: macrophages isolated from mice 12 h after *i.v.* injection with eNampt showed robust phosphorylation of STAT3 (**Fig. 2D**).

To establish causation for apoptosis, we knocked down STAT3 expression in macrophages using siRNA. As shown by the immunoblot in **Fig. 3A**, STAT3 knockdown was achieved using two separate Stat3 siRNA species, whereas scrambled siRNA did not decrease STAT3 expression. As in Fig. 1, lipoprotein-cholesterol-induced apoptosis was markedly suppressed by pre-treatment of the cells with eNampt (**Fig. 3B**, *left column of images*; quantified data in **Fig. 3C**). However, suppression of apoptosis by eNampt was largely abrogated in cells treated with the two active Stat3 siRNA species. With regard to additional controls for this experiment, scrambled siRNA did not reverse the survival effect of eNampt; none of the siRNA species induced apoptosis in non-cholesterol-loaded macrophages, either alone or plus eNampt; and the siRNA did not significantly alter cholesterol-induced apoptosis (data not shown). Almost identical results were obtained when apoptosis was induced by the UPR activator thapsigargin plus the SRA ligand fucoidan (**Fig. 3B**, *right column of images*; quantified data in **Fig. 3D**) and when the TUNEL assay was used for apoptosis instead of annexin V staining (data not shown).

Extracellular Nampt Induces STAT3 Phosphorylation by Promoting the Secretion of an Autocrine/Paracrine Stat3 Activator, Interleukin-6—eNampt could activate STAT3 through a direct effect on macrophages or by inducing the secretion of an autocrine/paracrine activator, which would then secondarily activate STAT3. We considered the latter possibility, because when eNampt was added directly to macrophages, the onset of STAT3

phosphorylation was delayed for at least 4 h (see Fig. 2A). To test the autocrine/paracrine hypothesis, conditioned medium (CM) was collected from control and eNampt-treated macrophages ("donor" macrophages) and then added to "recipient" macrophages for various periods of time (**Fig. 4A**). The data show that CM from eNampt-treated donor macrophages (eNampt-CM) induced STAT3 tyrosine phosphorylation in recipient macrophages after as little as 15 min of incubation. This result could not be explained by a direct effect on the recipient cells of residual eNampt in the CM, because, as mentioned above, eNampt takes at least 4 h to activate STAT3. This point was proven in a subsequent experiment in which the donor macrophages were incubated with eNampt for 5 h, then chased in eNampt-free medium for 12 h. The 12-h chase CM was also able to activate STAT3 in recipient macrophages after only 30 min of incubation (**Fig. 4B**). The ability of eNampt to induce the secretion of the STAT3-activating factor was dependent on new protein synthesis in the donor macrophages, because CM from macrophages treated with eNampt in the presence of the protein synthesis inhibitor cycloheximide did not induce STAT3 phosphorylation in recipient macrophages (data not shown).

We undertook a candidate approach to identify the active factor(s) in eNampt CM. Two STAT3-activating cytokines known to be secreted by macrophages are IL-10 and IL-6 (48), and IL-6 can be induced by Nampt (25). We found that eNampt induced the secretion of both of these cytokines (**Fig. 4C**). We then tested the ability of neutralizing antibodies against the IL-10 receptor and IL-6 receptor to block the ability of eNampt-CM to activate STAT3 in recipient macrophages. As shown by the data in **Fig. 4D**, the IL-10R antibody had no effect, while the IL-6R antibody markedly suppressed STAT3 phosphorylation. In a control experiment, we showed that the IL-10R antibody was effective at blocking STAT3 phosphorylation in macrophages incubated with recombinant IL-10 (data not shown). To further prove the role of IL-6 as the critical STAT3-activating factor in eNampt CM, we showed that anti-IL-6 antibody was also able to block STAT3 phosphorylation in recipient macrophages incubated with eNampt-CM (**Fig. 4E**).

To establish that this eNampt/IL-6 pathway is important for eNampt-mediated survival of ER-stressed macrophages, the effect of eNampt on macrophage apoptosis was tested in the absence or presence of neutralizing IL-6 antibodies, IL-10 antibodies, or both. As shown in **Fig. 4F** (*Experiment #1*), the survival effect of eNampt was substantially abrogated by the anti-IL-6 antibody but not by the anti-IL-10 antibody. Very similar results were obtained when apoptosis was assayed using the TUNEL assay instead of the annexin V assay (data not shown). A repeat experiment with two additional controls is shown in *Experiment #2* in **Fig. 4F**. Note that anti-IL6 had no significant effect on apoptosis when added to cholesterol-loaded cells in the absence of eNampt and that non-immune IgG had no effect on eNampt-treated, cholesterol-loaded cells. In a separate experiment, we found that conditioned medium from eNampt-treated donor cells did not decrease expression of the pro-apoptotic UPR effector CHOP in recipient macrophages (data not shown), indicating that eNampt-induced survival was not mediated through

CHOP suppression. In summary, eNampt activates STAT3 in macrophages by first inducing the secretion of IL-6, which then secondarily activates STAT3 through interaction with the IL-6 receptor. This pathway is critical for the ability of eNampt to block UPR-induced apoptosis.

Extracellular Nampt-Induced STAT3 Phosphorylation Does Not Require Nampt Enzymatic Activity—Two recent reports have shown that signaling/cytokine-like activities of eNampt require its enzymatic activity (20, 26), but most other studies in this area have not addressed this critical issue. To explore this point in the context of eNampt-induced STAT3 activation in macrophages, we first determined whether depletion of the Nampt substrate nicotinamide in the medium could block eNampt-induced STAT3 phosphorylation. We found that eNampt induced STAT3 phosphorylation similarly in macrophages incubated in control *vs*. nicotinamide-free medium (**Fig. 5A**). Moreover, STAT3 phosphorylation was not induced by the product of the nicotinamide phosphoribosyltransferase, nicotinamide mono-nucleotide (NMN) (**Fig. 5B**). Note that NMN can mimic the effect of eNampt on glucose-stimulated insulin secretion, a scenario in which enzymatic activity of eNampt is required (20). Third, the nicotinamide phosphoribosyltransferase inhibitor FK866 did not block the ability of eNampt to stimulate the phosphorylation of STAT3 (**Fig. 5C, *left blot***). As a positive control for the inhibitory action of FK866, we took advantage of the finding of Busso *et al*. (26) that LPS-induced IL-6 requires endogenous Nampt and is thus inhibited by FK866 (see Discussion). As expected from this finding, LPS also activated STAT3, and this effect was markedly blocked by FK866 (**Fig. 5C, *right blot***). These data also highlight another important point, namely, that the ability of eNampt to trigger IL-6/STAT3 signaling in the macrophages in the current study cannot be explained by contaminating endotoxin. This point is further substantiated by the following observations: (a) no other molecules were detected by mass spectrometric analysis of our Nampt preparation; (b) endotoxin levels were below the limit of detection by the *Limulus* amebocyte lysate assay, and similar results in terms of STAT3 activation and macrophage survival were found with recombinant eNampt made in HEK-293 cells instead of in *E. coli*; (c) eNampt led to STAT3 phosphorylation equally well in wild-type *vs*. LPS-resistant *Myd88-/-* macrophages; and (d) treatment of macrophages with LPS did not mimic the pro-survival action of eNampt in ER-stressed macrophages (data not shown).

As another test of the idea that eNampt-induced STAT3 pathway described here does not involve Nampt enzymatic activity, we used a series of Nampt mutants that have varying degrees of enzymatic activity (44). The data in **Fig. 5D** show that there was no correlation between eNampt enzymatic activity and its ability to activate STAT3. In particular, R392A Nampt, which retains approximately a third of Nampt enzymatic activity, was almost completely unable to activate STAT3 or to stimulate the secretion of IL-6 (**Fig. 5D-E**). Conversely, H247E, D219A, and S200D Nampt, which have very low enzymatic activity, were potent stimulators of STAT3 phosphorylation (**Fig. 5D**). In summary, the combined data in Fig. 5 indicate that the nicotinamide phosphoribosyltransferase activity

of eNampt is not needed to activate the IL-6/STAT3 pathway in macrophages.

Extracellular Nampt is Associated with Increased Levels of Specific Cell-Survival Proteins in Cholesterol-Loaded Macrophages—STAT3 activation has been linked to cell survival and the induction of cell–survival proteins in certain models of apoptosis (46). We therefore hypothesized that eNampt pre-treatment of cholesterol-loaded macrophages would increase the level of one or more cell-survival proteins, which could explain the cell-survival action of eNampt in these cells. In this context, we assayed the levels of five well-known macrophage survival proteins, apoptosis inhibitor in macrophages (AIM; SPα), Bcl-xL, Bcl-2, Mcl-1, and inhibitor of apoptosis proteins-1/2 (IAP1/2) (**Fig. 6**). The most important analysis involved comparing cholesterol-loaded macrophages ± eNampt to determine which proteins were increased by eNampt under conditions that eventually lead to apoptosis and protection by eNampt (3rd and 4th lanes of the immunoblots). Of the five proteins analyzed, AIM was markedly increased by eNampt under cholesterol-loading conditions, and Mcl-1 and IAP1/2 were modestly increased. The basal level of Bcl-2 was actually decreased by cholesterol-loading, and there was a slight restoration of Bcl-2 expression by eNampt treatment of cholesterol-loaded cells. Bcl-xL was not increased by eNampt under these conditions. In non-cholesterol-loaded cells, none of the cell-survival proteins were increased by eNampt. These data are consistent with the hypothesis that eNampt promotes the survival of cholesterol-loaded macrophages by increasing the expression of certain cell-survival proteins, particularly AIM.

DISCUSSION

A critical area of obesity research is centered on circulating proteins whose levels are altered by changes in body mass index. Understanding how these proteins affect obesity-associated diseases may provide invaluable clues to help stem the devastating consequences of the current obesity epidemic (14). One such protein, eNampt/PBEF/visfatin, has received much attention lately, but the biological effects of this molecule and its mechanisms of action are complex and incompletely understood. The biological effect studied here—promotion of macrophage survival—could represent an important process by which elevated levels of eNampt in obesity might influence obesity-associated disease (Introduction and below). With regard to mechanism, we have shown that the key signaling pathway that mediates the cell-survival effect of eNampt—autocrine/paracrine activation of STAT3—does not involve eNampt enzymatic activity.

We provide four independent pieces of evidence—including the use of inactive eNampt mutants—to support the conclusion that nicotinamide phosphoribosyltransferase activity is not needed to activate the STAT3 pathway. In theory, the IL-6/STAT3/cell-survival pathway shown here could have been caused by a contaminating molecule in our preparations of eNampt. However, we subjected our preparation of eNampt to mass spectrometric analysis, and only one molecule—eNampt—was detected (data not shown). Moreover, the IL-6 effect could not be explained by contaminating endotoxin, as described in Results. Rather, we hypothesize that eNampt stimulates

secretion of IL-6 by interacting with a yet-to-be-identified signaling receptor, although not the insulin receptor (see text and Ref. (28)). In a preliminary experiment, we have shown that macrophages subjected to Proteinase K-mediated cell-surface proteolysis no longer respond to eNampt in terms STAT3 activation (data not shown). However, identification of an "eNampt receptor," if it does exist, will require a major new undertaking.

While it may seem implausible that cells secrete an enzyme that acts non-enzymatically in triggering cell signaling, previous work has shown that the extracellular form of phosphoglucose isomerase induces cellular migration through interaction with a seven-transmembrane domain receptor (49). Intriguingly, the only Nampt mutant that lacked STAT3-activating activity was R392A, suggesting that R329 somehow plays a role in either cell interaction or its downstream effects. The fact that the S200D mutant retained activity may also be instructive. This mutant cannot form dimers, which is the predominant form of enzymatically active wild-type Nampt (44). Thus, while the dimer form is necessary for enzymatic activity, the monomer form may retain cytokine stimulating activity.

The two mechanistic areas that will require further study include how eNampt triggers IL-6 secretion and how STAT3 activation promotes survival in ER-stressed macrophages. A previous study showed that an inhibitor of the MAP kinase p38 could block eNampt-induced IL-6 mRNA and IL-6 protein secretion from human monocytes (25). However, there were no data in this study addressing the involvement or lack thereof of eNampt enzymatic activity. In terms of the cell-survival effect of STAT3, there is evidence of increased apoptosis in *Stat3*-/- mice, and STAT3-induced survival in cultured cells has been associated with induction of a number of cell-survival molecules, including Bcl-2, Bcl-x_L, Mcl-1, and survivin (46). The data in Fig. 6 show that several cell-survival proteins, particularly AIM, are increased in eNampt pre-treated cholesterol-loaded macrophages. Future experiments in which STAT3 and the cell-survival proteins are silenced will be needed to definitively establish the hypothesized pathway, namely, eNampt $\rightarrow$ STAT3 $\rightarrow$ AIM/Mcl-1/IAP $\rightarrow$ cell-survival. Beyond the cell-survival effect of STAT3 signaling, Nowell *et al*. (50) suggested that IL-6/STAT3 signaling can actually induce Nampt itself, which, if applicable to macrophages, might represent an amplifying positive feedback loop.

In terms of the biological endpoint investigated here, how might enhancement of macrophage survival by eNampt have an impact on obesity-related processes? Macrophage apoptosis has been proposed to be one mechanism of curbing inflammation after an initial host defense response (51). Prolonged survival of these cells, therefore, may bolster the inflammatory response known to be important in obesity (1-3), particularly in the setting of ER stress, where the inflammatory response is enhanced (52-55). In addition, the autocrine/paracrine factor that initiates the STAT3-mediated cell-survival pathway—IL-6—directly promotes inflammation (56). As mentioned above, an enzymatic Nampt pathway enhances IL-6 secretion in LPS-activated macrophages (26), and

this process could complement the non-enzymatic pathway shown.

In another scenario, prolonged macrophage survival has been shown to promote the early progression of macrophage-rich atherosclerotic lesions (9, 10), another hallmark of obesity. This concept is not to be confused with the pro-necrotic effect of macrophage apoptosis in advanced plaques, where efferocytosis is defective (11, 12). Even in advanced lesions, however, simply promoting macrophage survival may result in detrimental effects associated with *living* macrophages in these plaques, such as secretion of inflammatory cytokines, matrix proteases, and tissue factor (57).

A third possible link is related to the known role of IL-6/STAT3 signaling, including anti-apoptotic pathways, in promoting cancer (58), another obesity-associated disease (5). In particular, an eNampt-mediated survival effect on tumor cells, or, apropos to the current study, tumor-associated macrophages (TAMs) might promote tumor development (6). Interestingly, TAMs have a particularly important role in prostate cancer, one of the major cancers associated with obesity (5, 59). If so, these effects of eNampt may complement an NAD-mediated survival effect of enzymatically active Nampt on tumor cells themselves (60).

Whether or not macrophage survival and the hypothesized consequences are increased in obesity in an eNampt-dependent manner must await the creation of conditional eNampt-null mice, because *Nampt*-/- mice die *in utero* (20). However, the concentration of eNampt used in our cell culture studies is in the range of eNampt measured in the circulation of obese mice and humans (21-24). Indeed, studies in humans have shown positive correlations among obesity, plasma eNampt levels, and inflammatory markers, including IL-6 (14, 25). Moreover, we showed that intravenous injection of mice with eNampt activates STAT3 in peritoneal macrophages (Fig. 2D). In addition, locally synthesized eNampt, including that in adipose tissue (20) and atherosclerotic lesions (61), may result in high local concentrations of the protein.

In summary, eNampt stimulates an IL-6/STAT3-mediated cell-survival pathway in macrophages through a non-enzymatic mechanism. These actions may account for alterations in macrophage physiology in the setting of obesity, where eNampt levels are elevated. The consequences of such macrophage alterations could mediate the effects of obesity in inflammation, atherogenesis, and tumorogenesis. Future genetic manipulations of eNampt in mice will be needed to test these ideas *in vivo*.

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FOOTNOTES

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[3]The abbreviations used are: ACAT, acyl-CoA:cholesterol acyl transferase; BSA, bovine serum albumin; CHOP, C/EBP-homologous protein; CM, conditioned medium; eNampt, extracellular Nampt; ER, endoplasmic reticulum; IL, interleukin; JAK, Janus kinase; LDL, low-density lipoprotein; Nampt, nicotinamide phosphoribosyltransferase; LPS, lipopolysaccharide; NMN, nicotinamide mononucleotide; PBEF, pre-B cell colony-enhancing factor; PRR, pattern recognition receptor; STAT3, signal transducer and activator of transcription-3; TAMs, tumor-associated macrophages; UPR, unfolded protein response.

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FIGURE LEGENDS

FIGURE 1. **eNampt protects macrophages from ER stress-induced apoptosis. *A*,** Macrophages were pre-incubated in the absence or presence of 100 ng/ml eNampt for 24 h and then incubated with medium alone (*Control*) or medium containing eNampt; 50 μg/ml acetyl-LDL + 10 μg/ml 58035 (*Chol*); or eNampt plus acetyl-LDL/58035 for 20 h. Apoptosis was assayed by Alexa-488 annexin V staining (*green*). One representative image per condition is shown. The *graph* to the right of the images shows quantification of the apoptosis data (mean ± SEM, n = 4 fields of cells). *P*<0.001 for Chol *vs*. eNampt/Chol. ***B*,** Macrophages were pre-incubated in the absence or presence of 100 ng/ml eNampt for 24 h and then incubated with medium alone (*Control*) or medium containing eNampt; 20 μg/ml 7-ketocholesterol (7-KS); eNampt plus 7-ketocholesterol; 5 μg/ml tunicamycin plus 25 μg/ml fucoidan (*TN/Fuc*); eNampt plus tunicamycin/fucoidan; 5 μg/ml staurosporine (*STS*); or eNampt plus staurosporine. The incubations times were 18 h for the 7KC experiment, 24 h for the TN/Fuc experiment, and 16 h for the STS experiment. Apoptosis was assayed and quantified as described above. *P*<0.001 for 7KC *vs*. eNampt/7KC; TN/Fuc *vs*. eNampt/TN/Fuc; and STS *vs*. eNampt/STS.

FIGURE 2. **eNampt activates tyrosine phosphorylation of STAT3. *A*,** Macrophages were incubated in the absence (*Con*) or presence of 100 ng/ml eNampt for the indicated times. Whole-cell lysates were immunoblotted for tyrosine phosphorylated STAT3 (*pY-STAT3*) and total STAT3. ***B*,** Macrophages were treated with the indicated concentrations of eNampt or control medium for 24 h and then incubated under control or cholesterol (*Chol*) loading conditions for 5 h. Whole-cell lysates were immunoblotted for pY-STAT3 and β-actin. ***C*,** Macrophages were incubated for 18 h with 100 ng/ml eNampt ± 10 μg/ml AG490. Whole-cell lysates were immunoblotted for pY-STAT3 and β-actin. ***D*,** Male C57/B6L mice (3 per group) were injected *i.v.* with 5 μg of eNampt in 0.5 ml PBS or PBS alone (vehicle control). Twelve hours after this injection, peritoneal macrophages were collected and lysed in RIPA buffer. Equal amounts of cell lysates were blotted for pY-STAT3 and total STAT3.

FIGURE 3. **eNampt-induced survival of ER-stressed macrophages requires STAT3. *A*,** Immunoblot of STAT3 in control macrophages and macrophages incubated for 60 h with two different Stat3 siRNA species or scrambled siRNA. ***B*,** Annexin V fluorescence images are shown for macrophages that were first incubated for 60 h in the absence or presence of the indicated siRNA species and then pre-incubated in the absence or presence of 100 ng/ml eNampt for 24 h. The cells shown in the left column of images were incubated with medium alone or medium containing 50 μg/ml acetyl-LDL + 10 μg/ml 58035 (*Chol*) for 20 h, and those in the right column of images were incubated in medium alone or medium containing 0.25 μM thapsigargin + 50 μg/ml fucoidan (*Thaps/Fuc*) for 24 h. Apoptosis was assayed by Alexa-488 annexin V staining (*green*). ***C-D*,** Quantified apoptosis data for both cholesterol-induced and thapsigargin/fucoidan-

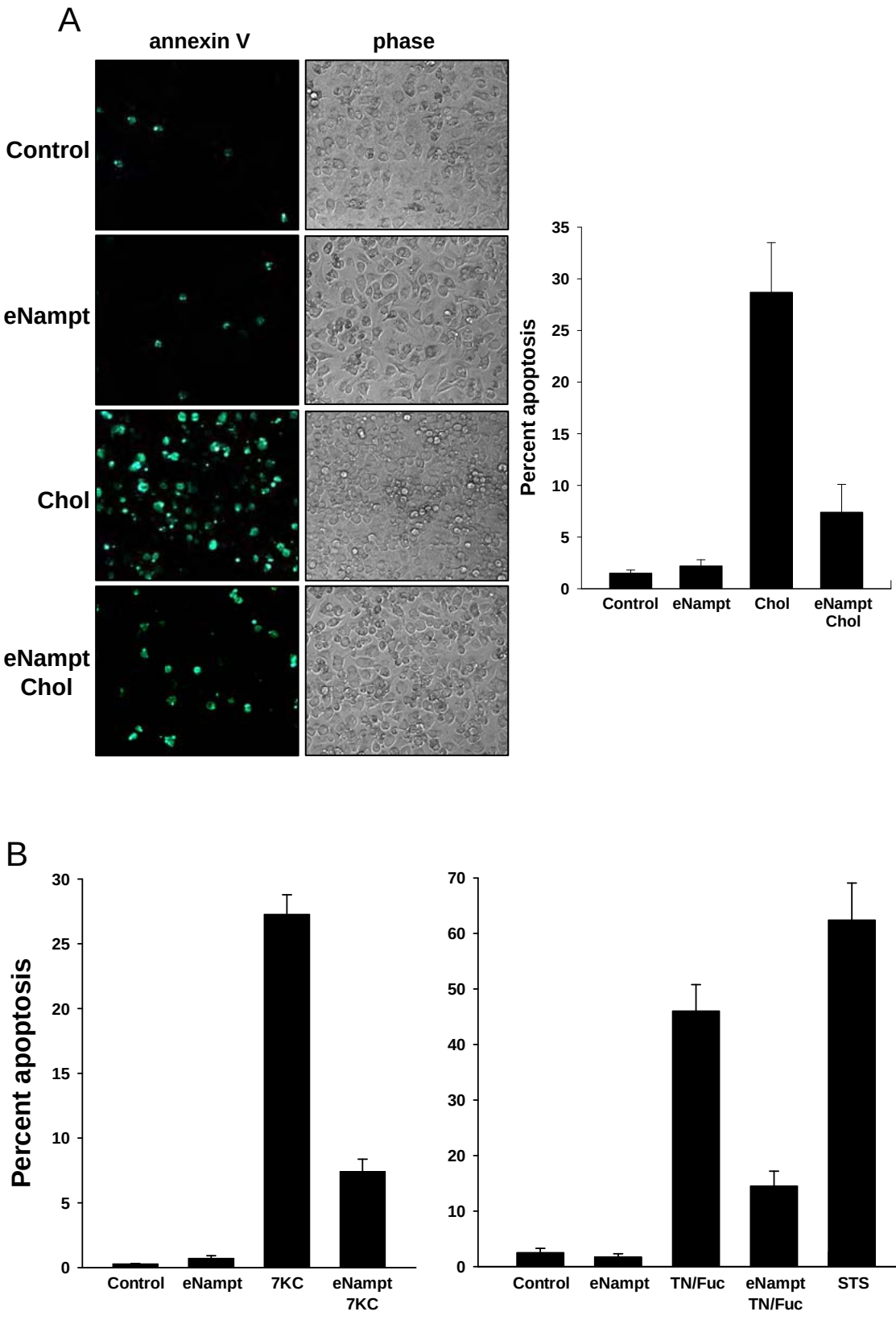
induced apoptosis (means ± SEM, n = 4 fields of cells). For both sets of apoptosis data, *P*<0.001 for the Nampt samples treated with the two Stat3 siRNA species *vs*. no siRNA or scrambled siRNA. When cholesterol loading or thapsigargin/fucoidan was omitted from the incubations, none of the siRNA species induced apoptosis (data not shown).

FIGURE 4. **eNampt induces STAT3 phosphorylation by promoting the secretion of an autocrine/paracrine STAT3 activator, interleukin-6. *A*, *Left blot*,** Macrophages were incubated in the absence (*Con*) or presence of 100 ng/ml eNampt (*eNmt*) for 24 h, and cell lysates were immunoblotted for pY- and total STAT3. ***Right blot*,** For these experiments, conditioned medium (*CM*) from control or eNampt-treated macrophages was transferred to fresh macrophages. The cells were then incubated in this medium for the indicated periods of time, after which cell lysates were immunoblotted for pY- and total STAT3. ***B*,** Macrophages were incubated in the absence or presence of 100 ng/ml eNampt for 5 h. The cells were washed and then incubated in fresh media for 12 h. This "chase" conditioned medium (*CM*) was then transferred onto fresh macrophages. After 30 min of incubation, cell lysates were immunoblotted for pY-STAT3 and β-actin. ***C*,** Macrophages were incubated in the absence or presence of 100 ng/ml eNampt for the indicated times. The culture media were collected and cleared of cell debris by centrifugation, and concentrations of IL-6 and IL-10 were measured by ELISA. ***D*,** eNampt-conditioned media were prepared as in *A* and then transferred to recipient macrophages that had been pre-treated for 30 min with 1 μg/ml anti-IL10 receptor IgG, anti-IL6 receptor IgG, or non-immune IgG control. The recipient cells were incubated for 30 min with the conditioned medium plus the respective IgGs. Cells were lysed, and the extracts were immunoblotted for pY-STAT3 and total STAT3. ***E,*** eNampt or control "chase" conditioned media (CM) were prepared as in *B*. The CM was incubated with or without 1 μg/ml anti-IL-6 antibody for 30 min. The media were then added to recipient macrophages and incubated for 30 min. 100 pg/ml recombinant IL-6, and IL-6 plus anti-IL-6 antibody, were used as positive and negative controls, respectively. Cells were lysed, and the extracts were immunoblotted for pY-STAT3 and β-actin. ***F*,** In both experiments shown, cholesterol-induced apoptosis was assayed in control or eNampt-pretreated macrophages exactly as in Fig. 1A, except some of the cells were incubated with 1 μg/ml anti-IL-6 antibody, anti-IL-10 antibody, or both throughout both the 24-h eNampt pre-treatment period and the 20-h cholesterol-loading period. In Experiment #1, *P*<0.01 for cholesterol *vs*. control and for the eNampt groups treated with anti-IL-6 or anti-IL-6 + anti-IL-10 antibody *vs*. no antibody or anti-IL-10 alone. In Experiment #1, *P*<0.01 for cholesterol *vs*. control and for the eNampt groups treated with anti-IL-6 *vs*. control IgG. There was no statistically significant difference among the cholesterol, eNampt/cholesterol/anti-IL-6, and cholesterol/anti-IL-6 groups.

FIGURE 5. **Induction of STAT3 phosphorylation by eNampt does not depend upon nicotinamide phosphoribosyltransferase enzymatic activity. *A*,** Macrophages were cultured in nicotinamide-free or

normal medium for 24 h. The cells were then incubated in the absence (*Con*) or presence of 100 ng/ml eNampt (*eNmt*) for 24 h, and cell lysates were immunoblotted for pY- and total STAT3. ***B*****,** Macrophages were incubated for 18 h with 100 ng/ml eNampt or 1.0 or 2.5 mg/ml NMN, and cell lysates were immunoblotted for pY- and total STAT3. ***C*****, *Left blot,*** Macrophages were incubated for 18 h with 500 nM FK866 alone, 100 ng/ml eNampt alone, or 100 ng/ml eNampt + 500 nM FK866. Whole cell lysates were immunoblotted for pY-STAT3 and total STAT3. ***Right blot,*** A similar experiment was conducted, except 100 ng/ml LPS was used instead of eNampt, based on the protocol of Busso *et al*. (26). ***D*****,** In the two experiments shown, macrophages were incubated for 18 h in the absence or presence 100 ng/ml of wild-type Nampt (WT) or the indicated Nampt mutants. Whole cell lysates were immunoblotted for pY-STAT3 and total STAT3. k_{CAT}/K_M data for each mutant, expressed as the percent of k_{CAT}/K_M for wild-type Nampt, is indicated above each lane. ***E*****,** Macrophages were incubated for 9 h in the absence or presence 50 or 100 ng/ml of wild-type Nampt (WT) or the R392A Nampt mutant. Cell culture supernatants were analyzed for the presence of IL-6 by ELISA (n=6 per condition).

FIGURE 6. **eNampt treatment of cholesterol-loaded macrophages increases the expression of specific cell-survival proteins.** Macrophages were pre-incubated in the absence or presence of 100 ng/ml eNampt for 24 h and then incubated with medium alone (*Con*) or medium containing eNampt; 50 μg/ml acetyl-LDL + 10 μg/ml 58035 (*Chol*); or eNampt plus acetyl-LDL/58035 for 6 h. Cell extracts were then assayed by immunoblot for the indicated cell-survival protein and β-actin as a loading control.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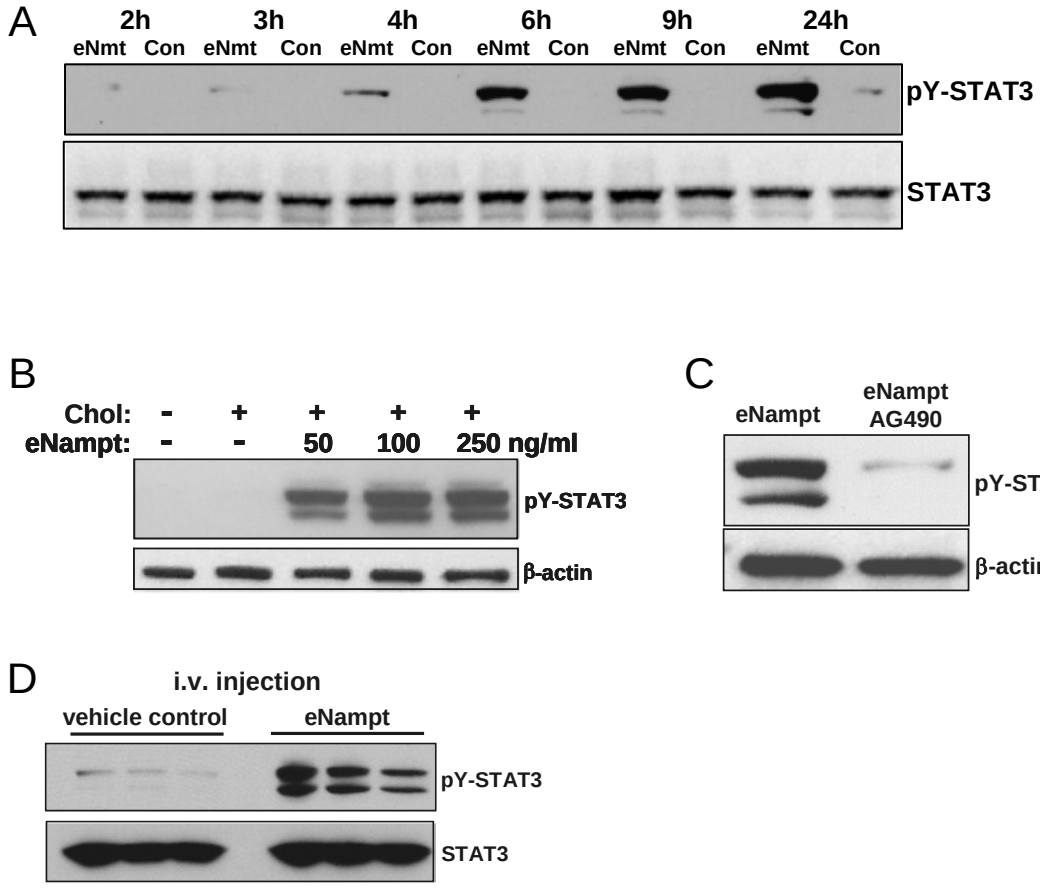
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Chol: - + + + +
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pY-STAT3
β-actin
C
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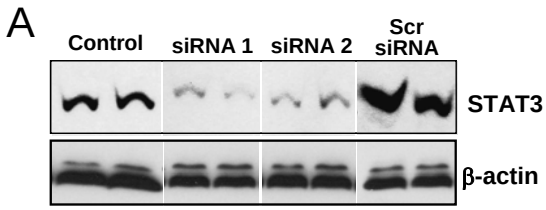
STAT3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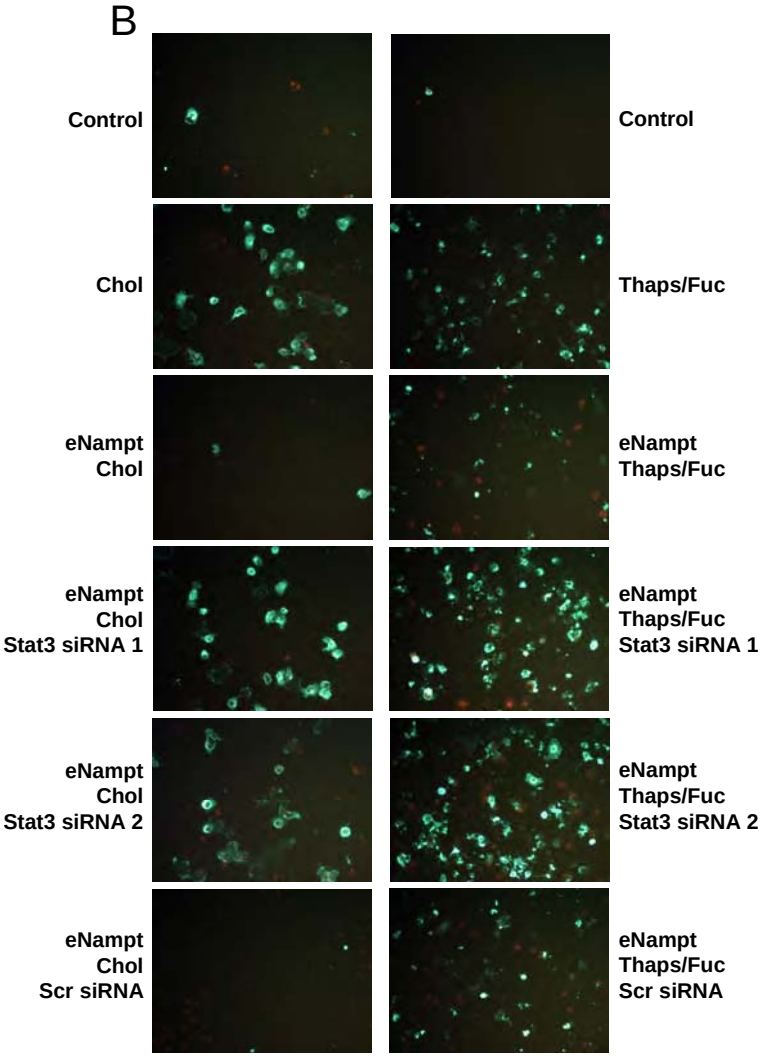
STAT3
β-actin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Thaps/Fuc
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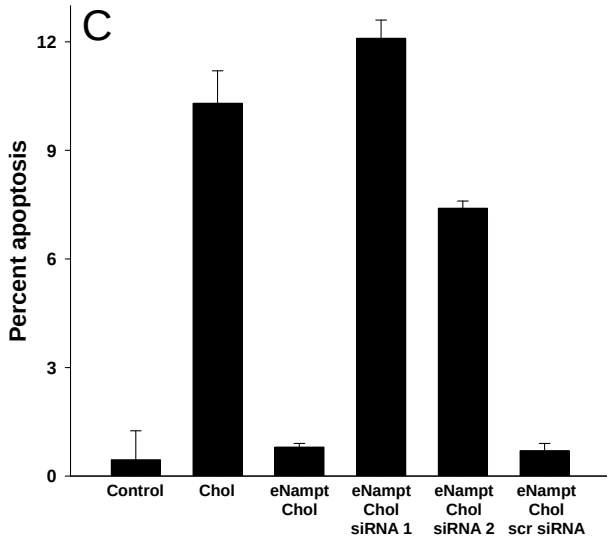
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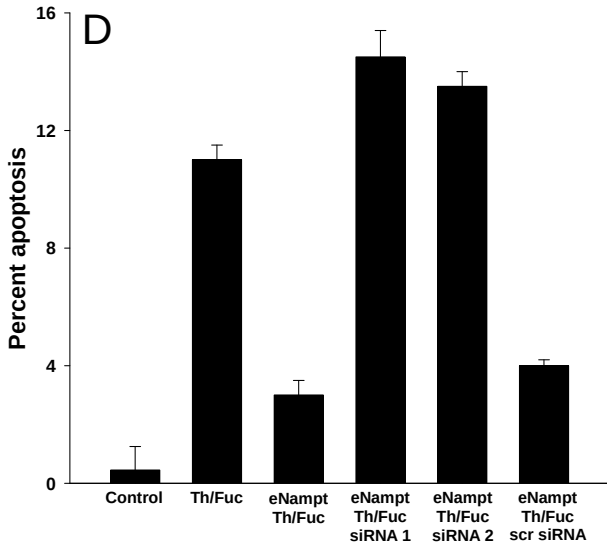
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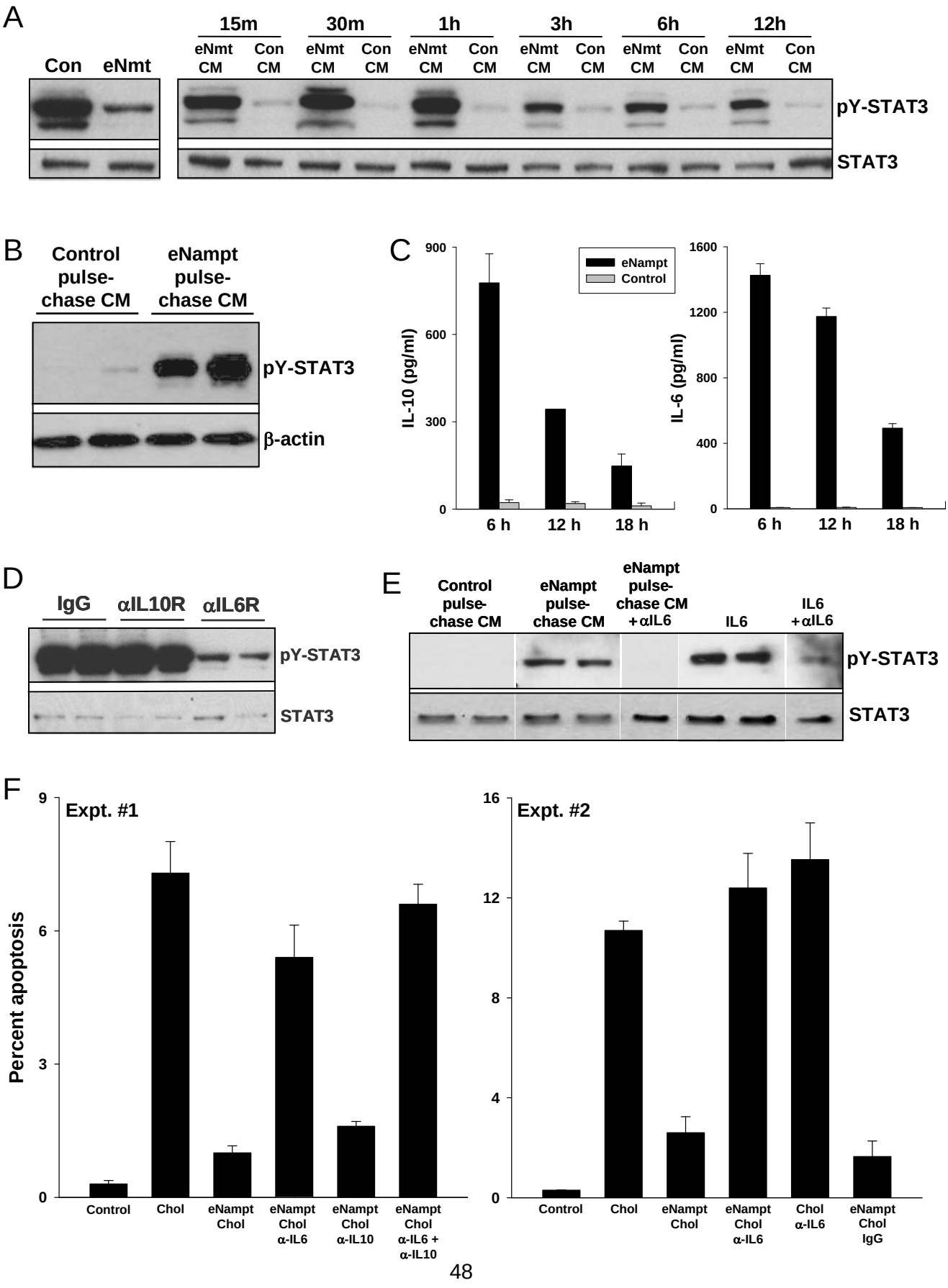
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pY-STAT3
β-actin
C
eNampt
Control
IL-10 (pg/ml)
IL-6 (pg/ml)
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12 h
18 h
D
IgG
αIL10R
αIL6R
pY-STAT3
STAT3
E
Control pulse-chase CM
eNampt pulse-chase CM
eNampt pulse-chase CM +αIL6
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pY-STAT3
STAT3
F
Expt. #1
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eNampt Chol
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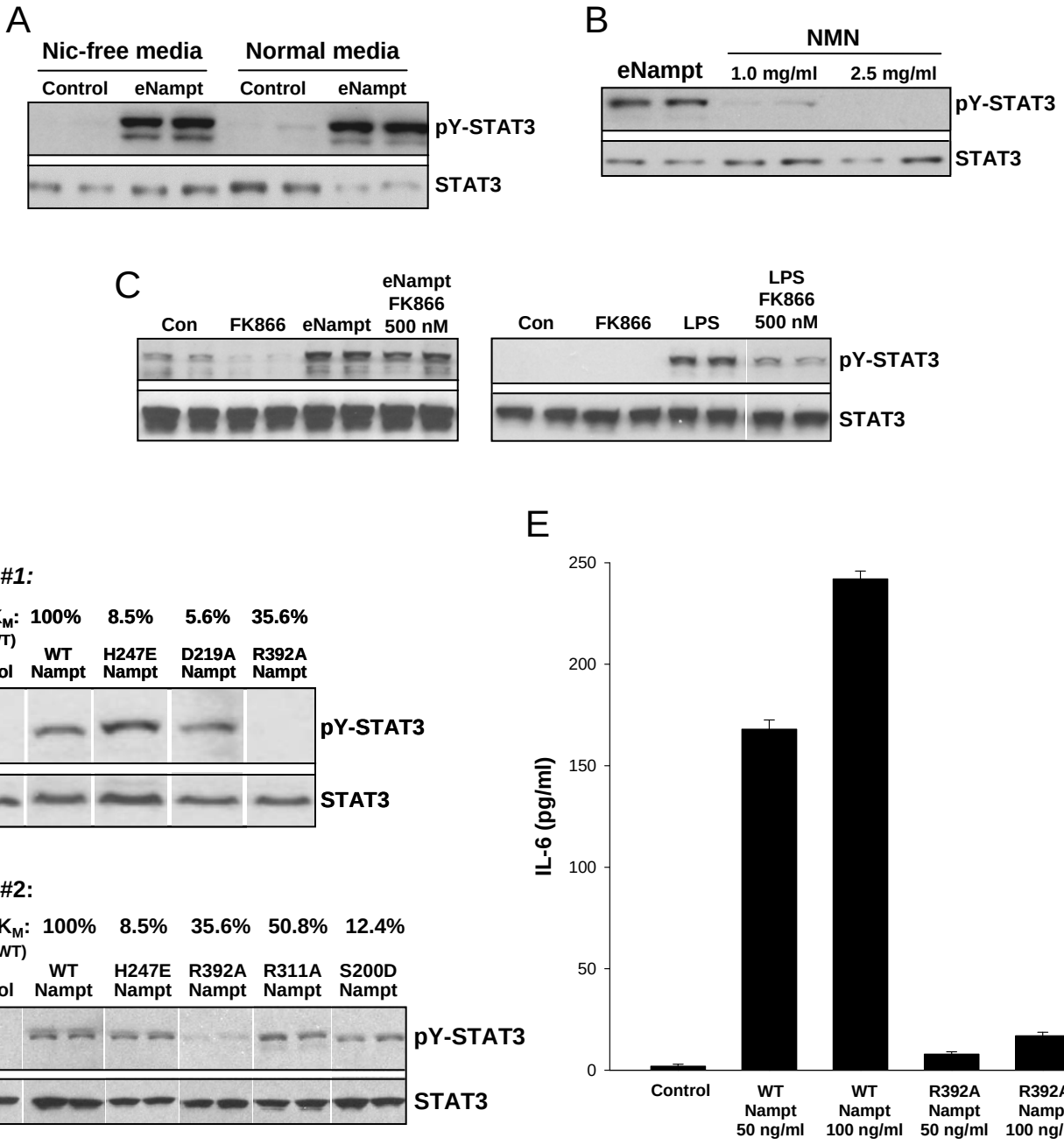
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Con
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LPS FK866 500 nM
pY-STAT3
STAT3
D
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kCAT/KM: (% of WT)
100%
8.5%
5.6%
35.6%
Control
WT Nampt
H247E Nampt
D219A Nampt
R392A Nampt
pY-STAT3
STAT3
Expt. #2:
kCAT/KM: (% of WT)
100%
8.5%
35.6%
50.8%
12.4%
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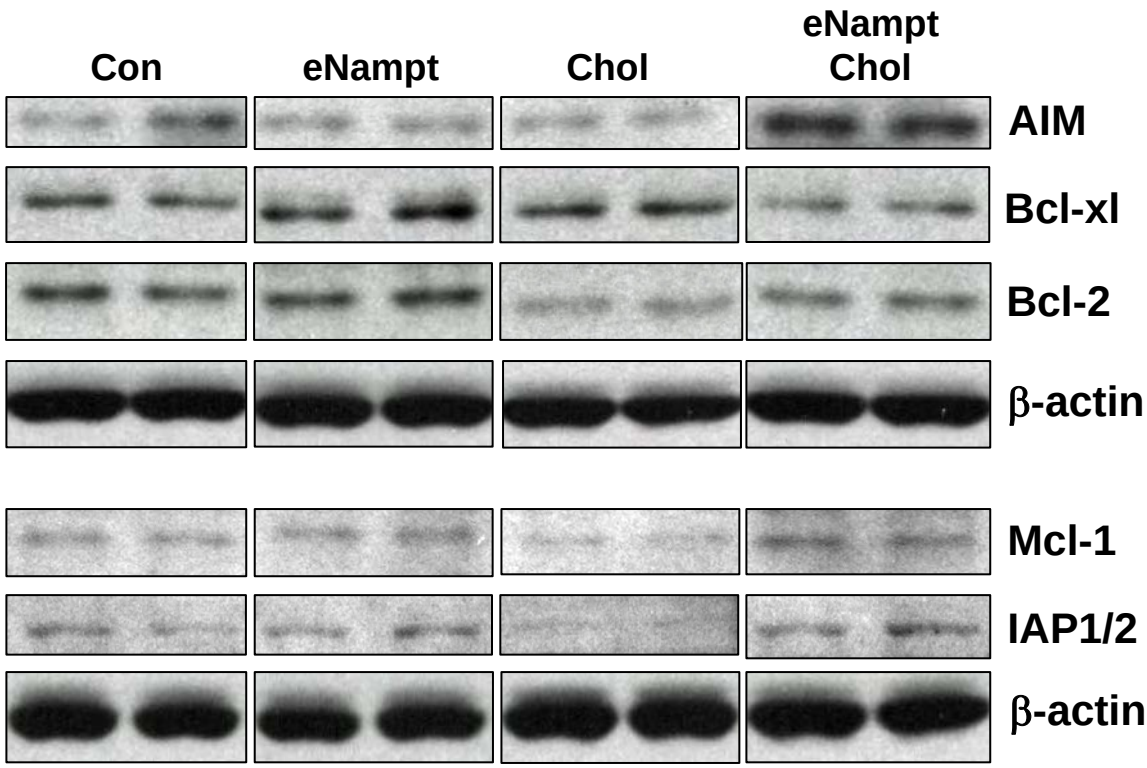
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Macrophage Apoptosis in Atherosclerosis: Consequences on Plaque Progression and the Role of Endoplasmic Reticulum Stress

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ABSTRACT

Atherothrombotic vascular diseases, such as myocardial infraction and stroke, are the leading cause of death in the industrialized world. The immediate cause of these disease is acute occlusive thrombosis in medium-sized arteries feeding critical organs. Thrombosis is triggered by the rupture or erosion of a minority of atherosclerotic plaques that have advanced to a particular stage of "vulnerability." Vulnerable plaques are characterized by certain key features, such as inflammation, thinning of a protective collagenous cap, and a lipid-rich necrotic core consisting of macrophage debris. A number of cellular events contribute to vulnerable plaque formation, including secretion of pro-inflammatory, pro-coagulant, and proteolytic molecules by macrophages as well as death of macrophages, intimal smooth muscles cells, and possibly endothelial cells. The necrotic core in particular is a key factor in plaque vulnerability, because the macrophage debris promotes inflammation, plaque instability, and thrombosis. Plaque necrosis arise from a combination of lesional macrophage apoptosis and defective clearance of these dead cells, a process called efferocytosis. This review will focus on how macrophage apoptosis, in the setting of defective efferocytosis, contributes to necrotic core formation and how a process known to be prominent in advanced lesions—activation of ER stress signal transduction pathways—contributes to macrophage apoptosis in these plaques.

THE LIFE AND DEATH CYCLE OF THE MACROPHAGE IN ATHEROGENESIS AND CONSEQUENCES OF LESIONAL MACROPHAGE APOPTOSIS

Atherogenesis begins with the focal retention of plasma apolipoprotein B-containing lipoproteins by extracellular matrix molecules in focal areas of the subendothelial intima of medium-sized arteries (1). This event, which is essential for atherogenesis, triggers a maladaptive inflammatory response, which likely occurs after the retained lipoproteins have been modified by oxidation, aggregation, and other means. The inflammatory response is dominated by the influx of bone marrow-derived monocytes, which diapedese through the endothelium and take up resident in the intima (2,3). Once there, the monocytes differentiate into macrophages, which then ingest the retained and modified lipoproteins. In early through mid-stage atherogenesis, the most prominent macrophage process is foam cell formation, which describes the ingestion and metabolism of lipoprotein-derived cholesterol. Specifically, the lipoprotein-cholesterol is trafficked from late endosomes to the endoplasmic reticulum (ER), where the enzyme acyl-CoA:cholesterol acyl transferase (ACAT) esterifies the cholesterol to cholesteryl fatty acyl esters (CE) (4). The CE coalesces into membrane-bound cytoplasmic lipid droplets, which give the cells a foamy appearance by microscopy.

The functional consequence of early lesional foam cell formation on atherogenesis has been debated (5), but genetic studies in mice in which monocytes are depleted or monocyte entry into lesions is blocked suggest that these macrophages contribute to the progression of atherosclerosis (6). The mechanism may involve secretion of pro-atherogenic molecules, such as inflammatory cytokines and pro-retentive extracellular matrix molecules, by macrophage foam cells (1-3). The overall concept that early-to-mid-stage macrophages are pro-atherogenic has also been suggested by studies investigating the consequences of macrophage apoptosis in these lesions (7). Although macrophages are relatively long-lived cells, there is a finite incidence of macrophage apoptosis throughout atherogenesis. In studies in which early lesional macrophage apoptosis was blocked by genetic engineering in mice, the lesions became more cellular and atherogenesis was accelerated (8). The converse has been observed when early lesional macrophage apoptosis is enhanced, i.e., a decrease in lesion progression (9). These data further support the notion that

macrophages play a pro-atherogenic role and suggest that the turnover of macrophages by apoptosis limits lesion cellularity in early atherogenesis.

In advanced lesions, macrophage apoptosis increases, and the functional consequences appear to be markedly different from those in earlier lesions (7). In particular, there is increasing evidence that advanced lesional macrophage apoptosis is associated with the development of a key feature of so-called "vulnerable plaques," namely, plaque necrosis (10). "Vulnerable plaque" is a term used to describe the minority of atherosclerotic lesions that progress to the point where they can trigger acute, occlusive lumenal thrombosis, which is the terminal event that precipitates acute coronary syndromes like unstable angina, acute myocardial infarction, sudden cardiac death, and stroke. These vulnerable plaques are characterized not by their larger size but rather by certain key characteristics, such as plaque necrosis, a heightened state of inflammation, and thinning of the "protective" collagenous scar, or "fibrous cap," that forms in advanced atheromata (11). These properties render the plaques more prone to rupture or erosion, which then exposes the overlying lumenal blood to pro-coagulant and pro-thrombotic molecules in the necrotic intima. A number of cellular events contribute to vulnerable plaque formation, including secretion of pro-inflammatory, pro-coagulant, and proteolytic molecules by macrophages as well as death of macrophages, intimal smooth muscles cells, and possibly endothelial cells. However, plaque necrosis per se is thought to be particularly important, because it is thought to promote plaque disruption and lumenal thrombosis through activation of inflammatory signaling, proteolysis of fibrous cap collagen, and promotion of coagulation and thrombosis by cell-released proteins and lipids (12). In addition, the physical nature of the lipid-rich fibrous cap places mechanical stress on the fibrous cap that is thought to contribute to its disruption (13).

THE CRITICAL FUNCTION OF EFFEROCYTOSIS THROUGHOUT ATHEROGENESIS

Why does macrophage apoptosis decrease lesion cellularity and progression in early lesions but promote plaque necrosis in more advanced lesions? Once likely explanation is that the normal physiologic process of phagocytic clearance ("efferocytosis") of apoptotic cells is more efficient in earlier than later lesions (7,14). To understand this concept, it is important to define

the term "necrosis." At the cellular level, "necrosis" refers to a type of cell damage in which cellular membranes, including the plasma membrane, become leaky and organelles swell, resulting in cell death and leakage of cellular contents into the extracellular environment (15). The released cellular debris is a potent inducer of inflammation in neighboring immune cells, such as macrophages and T cells (16). Necrotic cell death is to be distinguished from apoptotic cell death, which is characterized by intact membranes and condensed organelles and which does not usually incite inflammation unless the cells are not rapidly cleared by phagocytes (below) (15). At the tissue level, "necrosis" refers to areas that have accumulated necrotic cell-derived debris, *e.g.*, "caseating necrosis" in tuberculosis lesions in the lung. For the purpose of this review on atherosclerosis, I shall refer to cell necrosis as "macrophage apoptosis" and tissue necrosis as "plaque necrosis."

The most common cause of cell necrosis *in vivo* is so-called post-apoptotic, or secondary, necrosis, which results from the lack of efficient phagocytic clearance ("efferocytosis") of apoptotic cells (17). In normal physiology, during which apoptosis occurs at a very high rate with estimates as high as hundreds of billions of cells per day, apoptotic cells are rapidly cleared by both professional and "non-professional" phagocytes. Importantly, the clearance of apoptotic cells elicits an active anti-inflammatory response in efferocytes (18). When efferocytosis is not efficient, as is thought to occur in disease states such as systemic lupus erythrematosis and advanced atherosclerosis (below), apoptotic cells become necrotic, as defined above, and an inflammatory response ensues (17).

The lack of plaque necrosis and inflammation associated with macrophage apoptosis in early atherosclerotic lesions implies efficient efferocytosis, although this has never formally been proven. In advanced lesions, the converse appears to be the case, and here there is supporting experimental evidence. In particular, Schrijvers and Martinet (19) meticulously scored advanced human lesions for free and phagocyte (macrophage)-associated apoptotic cells, which were identified by the terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) stain. In comparison to human tonsils, an organ with efficient efferocytosis, advanced atheromata had a larger number of non-macrophage-associated TUNEL-positive cells. Furthermore, there are now a number of molecular genetic causation studies in mice in which genes involved in efferocytosis, such as apolipoprotein E, transglutaminase, milk fat-binding globulin, and Mertk, have been targeted (20). In many of these models, disruption of

efferocytosis is associated with plaque necrosis and, when examined, an increase in the number of non-macrophage-associated TUNEL-positive cells. Thus, it is likely that a defect in efferocytosis in advanced atheromata, the cause of which is not yet known, leads to post-apoptotic macrophage necrosis and eventually plaque necrosis.

THE ROLE OF ENDOPLASMIC RETICULUM STRESS IN ADVANCED LESIONAL MACROPHAGE APOPTOSIS

As summarized above, advanced lesional plaque necrosis likely involves a combination of macrophage apoptosis and defective efferocytosis. Thus, understanding the mechanisms of these two processes is a critical goal in atherosclerosis research. In this section, I will discuss a key area related to mechanisms of advanced lesional macrophage apoptosis. For a discussion of possible mechanisms of defective efferocytosis, the reader is referred to previous reviews on this topic (7,14).

All cells possess a highly conserved an integrated set of pathways called the Unfolded Protein Response (UPR) or Integrated Stress Response (ISR) that are designed to protect the endoplasmic reticulum (ER) from a variety of physiologic and pathophysiologic perturbations (21,22). These perturbations include increased protein synthesis ("client load"); disturbances in ER calcium homeostasis; alterations in the physical property of the ER membrane bilayer, *e.g.*, by certain types of lipids; disturbances in the redox environment of the ER; and misfolded proteins. There are three major branches of the UPR, each with specific functions related to correcting the aforementioned perturbations of the ER. Of great interest, there is evidence that these branches also regulate the delicate balance between cell survival and apoptosis in cells (23,24). From a teleological viewpoint, one may imagine that evolution has designed the UPR to keep cells alive to enable the repair process to proceed but also to have a "fail-safe" mechanism to trigger cell death if the perturbation is irreparable. The branch of the UPR that has been associated most with apoptosis involves the UPR effector CHOP, or GADD153 (23). In particular, while short term induction of CHOP is an important element of the protective UPR, prolonged expression of CHOP can lead to cell death. Many mechanisms of CHOP-induced apoptosis have been explored in cultured cells, including generation of reactive oxygen species

and down regulation of Bcl-2 (25), but evidence for a dominant pro-apoptotic mechanism *in vivo* is lacking. UPR-induced apoptosis can also be coordinated with suppression of the other two branches of the UPR—the IRE1-XBP1 and ATF6 branches—which can, under certain circumstances, promote cell survival (24).

Austin and colleagues (26) raised the idea that the UPR may play a role in atherosclerosis by showing that homocysteine, a potentially pro-atherogenic molecule, causes ER stress-induced growth arrest in endothelial cells. Our group was interested in mechanisms of advanced lesional macrophage apoptosis. Based on the finding that macrophages in advanced atheromata accumulate large amounts of FC (27), we used a model in which apoptosis of cultured macrophages was induced by loading the cells with large amounts of lipoprotein-derived unesterified, or "free," cholesterol (FC) (28-30). Under normal conditions, lipoprotein-derived FC is trafficked to the ER, where it is fatty acyl-esterified by the enzyme acyl-CoA:cholesterol acyl transferase (ACAT). FC accumulation in the macrophage model, therefore, requires inhibition or genetic targeting of ACAT (28). *In vivo*, a mouse model of advanced atherosclerosis in which macrophage ACAT was gene-targeted showed increase advanced lesional macrophage death (31), and several studies in which ACAT inhibitors were used in humans revealed worsening of atherosclerosis or heart disease in the drug-treated group (32,33).

In searching for mechanisms of FC-induced apoptosis, we made the observations that FC loading led to UPR activation and CHOP induction in a manner that depended upon cholesterol trafficking to the ER and that macrophages from *Chop*-/- mice showed ~70% protection from FC-induced apoptosis (34). Moreover, there was strong correlations among FC accumulation in the ER membrane, increased order parameter ("stiffening") of the normally liquid-disordered ("fluid") ER membrane, and inhibition of the ER calcium pump sarcoplasmic/endoplasmic reticulum calcium-dependent ATPase (SERCA) (35). Thus, a plausible hypothesis was one in which FC loading, through biophysical changes in the ER membrane, led to depletion of ER calcium through inhibition of SERCA, subsequent dysfunction of calcium-dependent protein chaperones, and eventual activation of the UPR secondary to chaperone dysfunction.

In vivo data supporting a role for the UPR in advanced lesional macrophage apoptosis comes from a number of studies. For example, aortic root lesions of *Apoe*-/- mice in which lipoprotein-cholesterol trafficking to the ER was compromised by a heterozygous mutation in the cholesterol trafficking protein NPC1 showed a decrease in advanced lesional macrophage apoptosis and

plaque necrosis (36). Austin and colleagues (37) found evidence of CHOP induction in the aortic root lesions of *Apoe*-/- mice, and we have recently observed a decrease in advanced lesional macrophage apoptosis and plaque necrosis in *Apoe*-/- mice that are also deficient in CHOP (E. Thorp, G. Li, and I. Tabas, manuscript in preparation). Most importantly, Myoishi *et al*. (38) found a striking correlation among ER stress markers, including CHOP; plaque vulnerability; and lesional apoptosis in samples of human coronary artery plaques. Together, these diverse pieces of experimental data support at least a partial role for UPR-induced apoptosis in the death of advanced lesional macrophages.

THE MULTI-HIT MODEL OF ER STRESS-INDUCED MACROPHAGE APOPTOSIS

We initially reasoned that FC-induced macrophage death was simply an example of UPR/CHOP-mediated apoptosis. Indeed, it is well known that if a very potent UPR inducer, such as high dose tunicamycin (a protein glycosylation inhibitor) or thapsigargin (a SERCA inhibitor), is added to cultured cells, apoptosis will ensue. However, ER stress *in vivo* is probably more subtle and, by itself, insufficient to cause apoptosis. Rather, ER stress would lower the threshold for one or more additional "hits" to trigger cell death. In that sense, we imagine that cells *in vivo* "sample" various sub-threshold noxious stimuli in their environment and trigger the "suicide" response of apoptosis only when the combination of these stimuli signals irreparable damage. Of interest in this regard, a number of compensatory cell-survival pathways, such as Akt, ERK, and NF-κB, are activated in the setting of ER stress, and some of the additional hits appear to function by suppressing these pathways (below).

In the case of apoptosis in ER-stressed macrophages, this "multi-hit" concept was initially supported by our observation that apoptosis induced by lipoprotein-derived FC could be markedly suppressed by the absence or inhibition of two cell-surface pattern recognition receptors (PRRs) that interact with atherogenic lipoproteins, the type A scavenger receptor (SRA) and toll-like receptor 4 (TLR4) (39,40). The inhibition of apoptosis in these experiments occurred despite unsuppressed UPR activation, indicating that the UPR alone was not sufficient for apoptosis. Moreover, apoptosis could be induced by combinations of a low-dose non-

lipoprotein ER stressor, such as thapsigargin, plus an SRA/TLR4 ligand such as fucoidan or acetyl-LDL, but not by either agent alone (40). Indeed, there is a long list of ER stressors and PRR ligands in atheromata (41), and a number of these combinations can induce macrophage apoptosis—completely in the absence of FC loading (40). Thus, the multi-hit model broadens the applicability of ER stress-induced macrophage apoptosis beyond the FC model. Examples of lesional ER stressors include oxidant stress, peroxynitrite, insulin resistance, glucosamine, saturated fatty acids, hypoxia, homocysteine, oxidized phospholipids, oxysterols such as 7-ketocholesterol, and serum starvation (41). Examples of lesional PRR ligands that trigger macrophage apoptosis during ER stress include lesion-modified forms of LDL, advanced glycation end-products (AGEs), β-amyloid, and oxidized phosphatidylcholine (41).

Beginning with the observation that combinations of sub-threshold ER stress plus PRR engagement could trigger apoptosis, further experimental work revealed a complex web of signal transduction pathways that lead to apoptosis (**Figure**). In this model, a combination of activation of pro-apoptotic signaling and suppression of compensatory cell-survival pathways eventually leads to cell death. Mechanistic studies have revealed several key hubs in this web of pathways as well as a number of integrated amplification loops. Two of the key hubs are calcium/calmodulin-dependent protein kinase II (CaMKII) and NADPH oxidase-mediated reactive oxygen species (ROS) (Refs. (42); J. Timmins, *et al.*, manuscript submitted; G. Li, *et al*., manuscript in preparation). Specifically, ER stress and CHOP, though various mechanisms, promotes ER calcium release and activation of CaMKII, which in turn activates a number of pro-apoptotic pathways, including JNK-mediated Fas induction, mitochondrial depolarization, STAT1 activation, and NADPH oxidase activation. ROS activation, in turn, amplifies this pathway through effects on calcium release and also by suppressing compensatory Akt-mediated cell-survival signaling (G. Li, *et al*. and T. Seimon *et al.*, manuscripts in preparation). Engagement of PRRs is necessary for apoptosis because it further contributes to STAT1 activation an also silences a cell-survival pathway mediated by interferon-β (40,42).

A remarkable feature of this network is that none of the pro-apoptotic steps by themselves are alone sufficient for apoptosis, which reflects the sub-threshold nature of the steps and, most importantly, the critical role of compensatory cell-survival signaling. This latter point was recently demonstrated in two *in-vitro* projects, one linking ER stress and efferocytosis and the other linking ER stress/PRR-mediated apoptosis with insulin resistance in macrophages. In the

first project, efferocytes that ingest FC-loaded apoptotic cells were found to be highly resistant to FC-induced death because NF-κB and Akt-mediated cell-survival pathways are activated in these cells (43). Conversely, macrophages with defective insulin signaling, a hallmark of type 2 diabetes, have suppressed NF-κB and Akt signaling. NF-κB suppression is mediated by increased nuclear localization of FoxO1, which induces that NF-κB inhibitor IκBε (44).

Different components of this integrated web have been tested *in vivo*. STAT1 deficiency in bone marrow-derived cells (macrophages in atheromata) in Western diet-fed *Ldlr*-/- mice suppresses advanced lesional macrophage apoptosis and plaque necrosis (42). Similar results have been obtained with CHOP deficiency (E. Thorp, G. Li, and I. Tabas, manuscript in preparation) and with deficiencies of two apoptosis-inducing PRRs, SRA and CD36 (Manning-Tobin, J.J., Moore. K.J., Seimon, T.A., Bell, S.A., Sharuk, M., Alvarez-Leite, J.I., de Winther, M.P.J., Tabas, I., Freeman, M.W., manuscript in revision). Two examples of how compensatory cell-survival signaling plays a role in vivo are *Apoe*-/- mice treated with pioglitazone, which suppresses the NF-kB cell-survival pathway in ER stressed macrophages (45), and *Apoe*-/- mice with macrophage-deficient p38α MAPK, which activates an Akt-mediated cell-survival pathway in ER stressed macrophages (Seimon *et al*., submitted manuscript). In both cases, the experimental mice showed increased advanced lesional macrophage apoptosis and plaque necrosis. Finally, in view of the increased sensitivity of insulin-resistant macrophages to ER stress-PRR-induced apoptosis (above), *Ldlr*-/- mice reconstituted with macrophages lacking insulin receptors—a proof-of-concept model of macrophage insulin resistance—showed increased advanced lesional macrophage apoptosis and plaque necrosis (46). This finding may have relevance to the rising epidemic of insulin resistance-associated atherothrombotic vascular disease, which is specifically associated with lesions having large necrotic cores (47).

SUMMARY AND CONCLUSIONS

Most individuals in industrialized societies have numerous atherosclerotic lesions in their coronary, carotid, and other susceptible peripheral sites arteries. Although only a small portion of these lesions will progress to plaques that have the potential to trigger acute occlusive lumenal thrombosis, the toll of this progression on morbidity and mortality in our society is huge.

Current cholesterol-lowering therapy has made a huge impact on lessening plaque progression and clinical events, but atherothrombotic vascular disease still remains the leading cause of death in the industrialized world. Indeed, the gains in cardiovascular disease prevention achieved by cholesterol lowering have been curtailed somewhat by the epidemic of obesity, insulin resistance, and type 2 diabetes, which are potent risk factors for atherothrombotic vascular disease and advanced plaque progression. In this context, complementary therapeutic approaches directed at the arterial wall in general, and plaque progression in particular, could have a tremendous effect on lessening disease burden, particularly given that type 2 diabetes will be an increasingly dominant force in promoting these processes for the foreseeable future. However, in order to achieve this goal, two major advances are needed: readily available and clinically useful imaging techniques that focus on advanced plaque morphology and a fundamental knowledge of the pathophysiology of plaque progression. Plaque progression involves a number of complex processes, including the topics of this review—ER stress-induced macrophage apoptosis and defective efferocytosis—but also including other forms of macrophage death, death of intimal smooth muscle cells and possibly endothelium, and roles played by living macrophages in advanced plaques. Within the narrow focus of this review, there is the hope that knowledge of how the combination of ER stress-induced macrophage apoptosis and defective late lesional efferocytosis can some day lead to a novel therapeutic strategy. For example, in other settings of ER stress-induced pathology, so-called chemical chaperones have been investigated as therapeutic agents (48). Likewise, strategies to enhance efferocytosis have been explored in the setting of systemic lupus erythrematosis, where defective clearance of apoptotic neutrophils in joints is thought to contribute to the disease (49). Coordinated developments in the areas of advanced plaque imaging and the cellular and molecular biology of plaque progression offer the best hope of realizing these goals.

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ABBREVIATIONS

CaMKII, calcium/calmodulin-activated protein kinase II; CHOP, CEBP-homologous protein; ER, endoplasmic reticulum; FC, free cholesterol; MAPK, mitogen-activated protein kinase; PRR, pattern recognition receptor; ROS, reactive oxygen species; SRA, type A scavenger receptor; STAT, signal transducer and activator of transcription; TLR, toll-like receptor; TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling; UPR, unfolded protein response.

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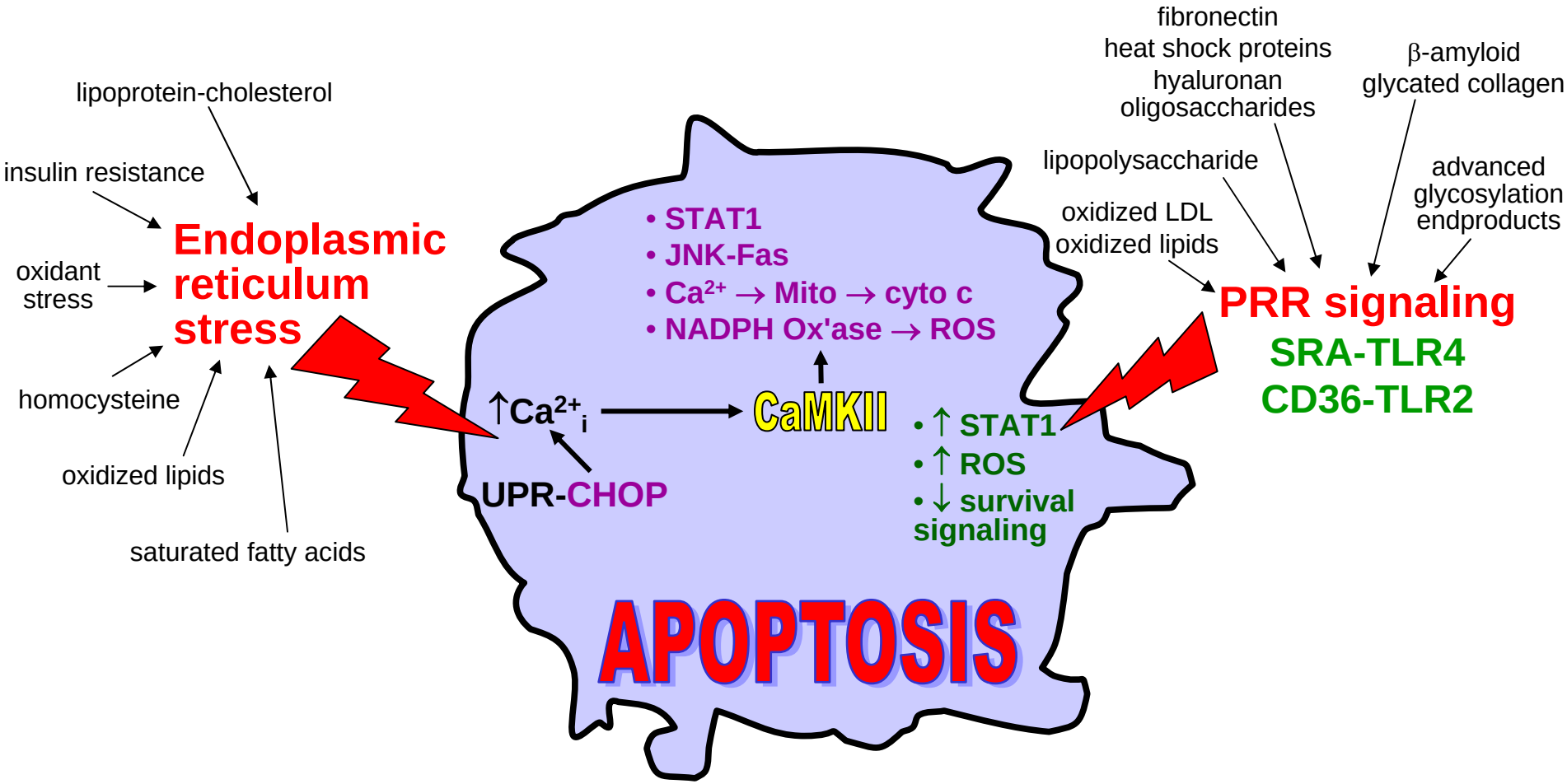
FIGURE LEGEND

FIG. 1. The ER stress-PRR model of advanced lesional macrophage apoptosis. Macrophages in advanced atheromata are exposed to a number of factors that induce ER stress and also to multiple ligands for pattern recognition receptors (PRRs). ER stress via CHOP induction and other mechanisms triggers ER calcium release. The resultant increase in cytosolic calcium, through activation of CaMKII, induces multiple pro-apoptotic pathways, including STAT1, JNK-Fas, calcium-induced mitochondrial depolarization, and NADPH oxidase-mediated ROS. When combined with combinatorial PRR signaling, such as SRA-TLR4 and CD36-TLR2, some of the pro-apoptotic pathways are amplified and some compensatory cell survival pathways are suppressed. The combination of all of these events tips the balance between sub-threshold pro-apoptotic signaling and compensatory cell survival signaling in favor of apoptosis. The key concept imbedded in this multi-hit model is that each of the pro-apoptotic hits are not, by themselves, sufficient to trigger apoptosis under the "subtle" conditions of the *in vivo* environment. Rather, the cells monitor the environment for a variety of noxious stimuli and do not trigger a suicide response unless the combination of these stimuli is deemed to indicate damage that is beyond repair. See text for details.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Endoplasmic reticulum stress
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CaMKII
UPR-CHOP
• ↑ STAT1
• ↑ ROS
• ↓ survival signaling
APOPTOSIS
fibronectin
heat shock proteins
hyaluronan oligosaccharides
β-amyloid
glycated collagen
lipopolysaccharide
advanced glycosylation endproducts
oxidized LDL
oxidized lipids
PRR signaling
SRA-TLR4
CD36-TLR2

Mechanisms and Consequences of Macrophage Apoptosis in Atherosclerosis

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ABSTRACT

Macrophage apoptosis is an important feature of atherosclerotic plaque development. Research directed at understanding the functional consequences of macrophage death in atherosclerosis has revealed opposing roles for apoptosis in atherosclerotic plaque progression. In early lesions, macrophage apoptosis limits lesion cellularity and suppresses plaque progression. In advanced lesions, macrophages apoptosis promotes the development of the necrotic core, a key factor in rendering plaques vulnerable to disruption and in acute lumenal thrombosis. The first section of this review will examine the role of phagocytic clearance of apoptotic macrophages—a process known as efferocytosis—in the dichotomous roles of macrophage apoptosis in early *vs*. advanced lesions. The second section will focus on the molecular and cellular mechanisms that are thought to govern macrophage death during atherosclerosis. Of particular interest is the complex and coordinated role that the endoplasmic reticulum (ER) stress pathway and pattern recognition receptors (PRRs) may play in triggering macrophage apoptosis.

CONSEQUENCES OF MACROPHAGE DEATH

Macrophages play crucial roles as a primary line of defense against infectious pathogens and foreign material and by ridding tissues of apoptotic debris. However, under pathological conditions, macrophages can promote a number of important disease processes, including insulin resistance, cancer, and

atherosclerosis (1;2). In the case of atherosclerosis, the topic of this review, a macrophage-dominant maladaptive inflammatory response develops as a reaction to the subendothelial retention and modification of apolipoprotein B-containing lipoproteins (3). In all stages of atherosclerotic lesions, activated macrophages, probably dominated by the "classically" activated M1 subset (4), secrete inflammatory cytokines and other molecules that contribute to lesion progression (5). Therefore, processes that increase macrophage accumulation in lesions, notably influx and proliferation, can promote lesion development, while those that decrease macrophage accumulation, such as apoptosis coupled with phagocytic clearance and macrophage egress, can retard lesion progression. In advanced lesions, macrophage apoptosis is not properly coupled with phagocytic clearance, and so in this setting macrophage death is associated with a detrimental role—plaque necrosis (6;7). This process leads to expansion of the necrotic core of advanced plaques, which contributes to plaque disruption and acute thrombosis (8). Thus, depending on the efficiency of apoptotic cell clearance, macrophage death can be a process that limits lesion cellularity or promotes plaque necrosis. In this review, we summarize the evidence supporting this dichotomous model of lesional macrophage death and discuss new concepts related to mechanisms of macrophage apoptosis and phagocytic clearance of apoptotic cells.

Macrophage death as a factor that limits lesion cellularity

Macrophage apoptosis occurs during all stages of atherosclerosis (9;10). Apoptotic cells have been identified in vivo using a variety of techniques, including annexin V staining, which is indicative of phosphatidylserine externalization; condensed nuclei; terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL), which signifies DNase-mediated DNA fragmentation; and caspase activation. Over the last few years, mouse models have been developed to explore the functional consequences of macrophage death in atherosclerosis. In relatively early lesions, *i.e.*, prior to necrotic core development, there is an inverse relationship between macrophage apoptosis and lesion size. For example, reconstitution of APOE*3-Leiden mice with p53-/- bone marrow resulted in reduced macrophage apoptosis, while macrophage content and lesion area were significantly increased (11). Another study demonstrated reduced macrophage apoptosis and increased lesion size in *Ldlr*-/- mice reconstituted with Bax-deficient bone marrow (12). The beneficial aspect of early lesional macrophage death has also been documented using mice deficient in the pro-survival molecule AIM (*a*poptosis *i*nhibitor expressed by *m*acrophages; also called Spα or Api6). This study found that *Aim*-/-;*Ldlr*-/- macrophages were more susceptible to oxLDL-induced apoptosis, and double knockout mice exhibited accelerated macrophage death and a significant reduction in early lesion area (13). Taken together, these findings support the

concept that macrophage apoptosis in early lesions is beneficial by suppressing lesion cellularity.

Macrophage death as a factor that promotes advanced plaque necrosis

As a prelude to this section, we wish to clarify the use of the term "necrosis" when referring to processes related to advanced atherosclerosis. On a cellular level, "necrosis" refers to a type of cell perturbation in which membranes become leaky and organelles swell, ultimately leading to cellular death. *In vivo*, cell necrosis can result when apoptotic cells, a programmed form of cell death in which membranes are initially intact and organelles are condensed, are not rapidly ingested by neighboring phagocytes. When this happens, the non-ingested apoptotic cells eventually become leaky and swollen. This type of cell death is often called "post-apoptotic," or "secondary," necrosis. At a tissue level, "necrosis" refers to collections of cell debris resulting from necrotic cell death. For example, in tuberculosis this process is referred to as "caseating necrosis." For the purpose of this review on atheromata, we refer to cell necrosis as "post-apoptotic macrophage necrosis" and tissue necrosis as "plaque necrosis". "Necrotic core" is often referred to in the literature as "lipid core," because the dying macrophages are filled with lipid, mostly cholesterol, which becomes incorporated as extracellular lipid into the areas of plaque necrosis.

Observational studies of advanced atherosclerotic lesions have shown that apoptotic macrophages accumulate in focal areas surrounding the developing necrotic core (14). Rather than simply "guilt by association," the fact that necrotic

cores contain predominantly macrophage debris has given rise to a concept alluded to above, namely, that plaque necrosis develops as a direct consequence of post-apoptotic macrophage necrosis (7;14). The necrotic debris is a source of proinflammatory stimuli and proteases and thus can elicit an inflammatory response and cause damage to nearby cells. These events, together with stresses on the fibrous cap caused by the physical nature of the necrotic core (15), can contribute to fibrous cap rupture, exposure of tissue factor, and subsequent lumenal thrombosis (7) **(Figure 1).**

To support this overall concept using a molecular and genetic approach, investigators have turned to mouse models in which proteins involved in macrophage apoptosis have been genetically altered. Because the traditional models of murine atherosclerosis, namely, Western diet-fed *Apoe*-/- and *Ldlr*-/- mice, do not develop plaque disruption or acute thrombosis, advanced lesional macrophage apoptosis and plaque necrosis are often used as endpoints to test causation.

As will be discussed in more detail below, endoplasmic reticulum stress (ER stress) is a likely factor that promotes advanced lesional macrophage death. Markers of ER stress have been shown to occur in advanced atherosclerotic lesions of both human and mouse, and thin or ruptured advanced human plaque have the highest accumulation of ER-stressed macrophages (16-19). These ER-stressed macrophages occur in areas of high TUNEL reactivity (18). Moreover, recent studies designed to look at the contribution of the ER stress pathway to macrophage apoptosis have shown a correlative relationship

between macrophage death and necrotic core development. For example, macrophages with haploinsufficiency of the cholesterol trafficking protein NPC1 are protected from apoptosis induced by unesterified cholesterol, an ER stress-inducing agent (16;20). When compared to *Apoe*-/- mice, *Npc1*+/-;*Apoe*-/- have a marked reduction in apoptosis and necrotic area (20). A similar reduction in macrophage apoptosis and plaque necrosis was observed using *Ldlr-/-* mice reconstituted with *Stat1*-/- bone marrow (21). STAT1 is a transcription factor that is phosphorylated and activated in human plaques and is necessary for ER stress-mediated macrophage apoptosis *in vitro* (21). As another example, thiazolidinediones (TZD's) have recently been shown to enhance ER stress-induced macrophage apoptosis *in vitro*. When administered to non-diabetic LDL receptor-deficient mice with pre-established non-necrotic lesions, there was a substantial increase in lesional macrophage apoptosis and enhanced plaque necrosis (22). In yet another example, in vitro studies have shown that macrophages with defective insulin signaling, including those from insulin-resistant mice, are more susceptible to ER stress-induced apoptosis (23). When *Ldlr*-/- mice were reconstituted with insulin receptor-deficient bone marrow as a proof-of-concept model of macrophage insulin resistance, an increase in advanced lesional macrophage apoptosis and plaque necrosis was observed. These combined data provide strong evidence in support of the hypothesis that macrophage apoptosis in advanced atheromata promote plaque necrosis.

The role of efferocytosis in modulating plaque necrosis

As described above, whether apoptosis leads to a decrease in cellularity or an increase in tissue necrosis depends to a large extent on the efficiency of apoptotic cell clearance by phagocytes, a process known as efferocytosis (24;25). In early atherosclerotic lesions, low levels of apoptotic macrophages suggest a normal or unperturbed efferocytic process (9). However in advanced lesions, the large number of apoptotic macrophages that accumulate almost certainly suggests perturbed efferocytosis even if the rate if apoptosis were also increased. Indeed, recent work by Schrijvers *et al*. has demonstrated that efferocytosis is defective in advanced lesions (6). In-vitro work has also shown that modified lipoproteins such as oxidized-LDL, which are abundant in advanced plaque, inhibit efferocytosis (26). Apoptosis itself may also be influenced by the stage of lesion progression. For example, as the necrotic core develops and cellular debris accumulates, the macrophage environment becomes enriched in proinflammatory cytokines and pattern recognition receptor ligands that, as discussed in more detail in the following section, may further enhance macrophage death. Therefore, necrotic core development likely occurs through the combination of defective efferocytosis and enhanced macrophage death (7). The importance of efferocytosis has recently been documented using in vivo mouse models that are deficient in efferocytic receptors. Two independent studies have shown that deficiency of Mertk, a phagocytic receptor for apoptotic cells, leads to a marked increase in apoptotic macrophages in lesions and

enhanced plaque necrosis (27-29). Similar results of enhanced apoptotic macrophage accumulation and necrosis were also shown with deficiencies of other molecules thought to play roles in efferocytosis, including apolipoprotein E, Fas, transglutaminase-2, complement protein C1q, and lactadherin (28)

MECHANISMS OF MACROPHAGE DEATH

Induction of macrophage apoptosis in atherosclerotic lesions likely involves the chronic, cumulative effect of several subtle, subthreshold "hits" rather than a single acute, catastrophic event. Examples of proapoptotic processes that occur in atheromata are oxidant stress (30), high concentrations of cytokines such as TNFα (31), unesterified cholesterol or oxysterols (16;18), oxLDL, activation of the Fas death pathway by Fas ligand (32), and ER stress (16-19) **(Figure 2)**. ER stress in particular, through activation of the Unfolded Protein Response (UPR), is strongly correlated with advanced lesional macrophage apoptosis and plaque necrosis in both murine lesions and in human coronary artery vulnerable plaques (16-19).

The roles of ER stress and pattern recognition receptors in advanced lesional macrophage apoptosis

The UPR exists as a means to protect cells from the accumulation and detrimental effects of misfolded proteins and therefore functions in normal physiology as an adaptive survival pathway (33). In particular, when misfolded proteins accumulate, a unique set of signal transduction events take place to

simultaneously halt further protein translation and up-regulate chaperones and transcription factors that serve to increase the capacity for the ER to process client proteins. When the amount of misfolded proteins exceeds the capacity for proper protein folding, or are not degraded through the ER-associated degradation (ERAD) pathway, apoptosis can ensue. Apoptosis in this context is highly dependent on prolonged expression of the UPR effector CHOP (Gadd153) (16;34).

There are many potential causes of ER stress in atherosclerotic plaques that are likely to influence advanced plaque progression. Examples of athero-relevant ER stress inducers include oxidant stress and peroxynitrite (30), insulin resistance (23), glucosamine (35), saturated fatty acids (36), hypoxia (37), homocysteine (38), oxidized phospholipids (17), oxysterols such as 7-ketocholesterol (18), serum starvation, and unesterified cholesterol accumulation from the uptake of modified, aggregated, and remnant lipoproteins (16;34). However, because ER stress is usually a protective response, high levels and prolonged activation of ER stress would be needed to induce apoptosis. A more likely scenario that may be occurring in advanced lesional macrophage apoptosis is that more physiologic levels of ER stress combine with one or more additional noxious “hits” to trigger apoptosis. In vitro work has supported this concept by showing that additional “hits” can enhance macrophage apoptosis during low levels of ER stress.

One category of atherosclerosis-relevant "second hits" that trigger apoptosis in macrophages undergoing low-level ER stress is engagement of pattern

recognition receptors (PRRs). PRRs are cell-surface receptors that bind pathogens, foreign antigens, endogenous proteins, and modified lipids through their ability to recognize a variety of structural or molecular motifs called pathogen-associated molecular patterns (PAMPs). Examples of PRRs include scavenger receptors and toll-like receptors (TLRs). When cells, particularly macrophages, bind PAMPs, inflammation and other processes are triggered as part of an innate immunity host defense mechanism.

Many endogenous PRR ligands have been found to accumulate in atherosclerotic plaques and have the potential to contribute to macrophage apoptosis by acting as "second hits" during ER stress. Examples of ligands that trigger macrophage apoptosis during ER stress include lesion-modified forms of LDL, advanced glycation end-products (AGE's), β-amyloid, and oxidized phosphatidylcholine, which engage scavenger receptors such as SRA and CD36 (34;39) (Seimon T. and Tabas I., unpublished data) **(Figure 2)**. Mechanistic studies have shown that scavenger receptors and toll-like receptors provide combinatorial proapoptotic signals that are necessary to trigger apoptosis in ER-stressed macrophages. Examples include the combination of SRA and TLR4 activation by SRA ligands (39) and the combination of CD36 and TLR2 activation by oxidized phospholipids (Seimon T. and Tabas I., unpublished data). In the case of the SRA-TLR4 pathway, apoptosis is dependent on SRA-mediated suppression of the macrophage survival protein interferon-β and on TLR4-mediated activation of the proapoptotic signal transducer STAT1 (21;39).

Two in vivo causation experiments have provided support for this model. The key role of macrophage STAT1, which is activated in advanced human coronary atheromata, was recently demonstrated to have a causal role in advanced lesional macrophage apoptosis and plaque necrosis in western diet-fed *Ldlr*-/- mice (21). More recently, we found that the combined deficiency of CD36 and SRA protected *Apoe*-/- mice from advanced lesional macrophage apoptosis and plaque necrosis (Tobin-Manning J., Moore K., Seimon T., and Tabas I., manuscript under revision). These data suggest that SRA and/or CD36 contribute to macrophage apoptosis and plaque necrosis in advanced lesions. Studies are currently underway to determine whether TLR2 or TLR4 can directly participate in macrophage apoptosis and necrotic core formation in vivo.

A possible teleology for induction of apoptosis in ER-stressed macrophages by PRR ligands

As mentioned above, ER stress is considered to be an adaptive pathway to protect macrophages from the increased burden of client protein overload. Thus, the ability to commit suicide during ER stress by simply engaging a subset of PRRs would seem counterproductive to the goal of keeping macrophages alive to fight infection. However, there are classes of infectious organisms that depend upon *living* macrophages to survive. Examples include viruses and intracellular bacteria, such *Mycobacterium tuberculosis* and *Brucella* species (40;41). Thus, the triggering of apoptosis could actually be part of the innate host defense system to prevent chronic infection by these organisms. In this regard,

there is evidence that intracellular organisms activate the UPR as a mechanism to support synthesis of pathogen proteins (41-43), and these organisms also display PAMPs that can activate scavenger receptors and TLRs. Most interestingly, in vitro studies have shown that macrophage apoptosis is associated with control of infection by *M. tuberculosis* (40), and genetic studies in mice have shown an association between resistance to infection by *M. tuberculosis* and induction of macrophage apoptosis (44). Recent work from our lab has shown that TLR-dependent macrophage apoptosis induced by *Mycobacterium* is enhanced by ER stress (Seimon T, Tabas I, and Nathan C, unpublished data). Although ER stress-PRR-induced macrophage apoptosis may be a highly detrimental process in advanced atherosclerosis—a post-reproductive disease with little or no evolutionary pressure—it may represent an evolutionarily conserved process that is important for host defense.

CONCLUSIONS AND FUTURE DIRECTIONS

Macrophage apoptosis in atherosclerotic lesions likely occurs through a combination of cellular stresses and events that differ depending on the stage of lesion progression. Apoptosis may be induced through a variety of ER stressors working alone or in combination with PRR ligands, or by the accumulation of additional cellular stresses such as oxidized lipids and death receptor ligands **(Figure 2)**. The consequence of macrophage apoptosis, whether beneficial by suppressing cellularity in early lesions, or detrimental by contributing to necrotic core formation in advanced lesion, is likely dependent on the efficiency of

lesional phagocytes to clear these dead cells. An important goal therefore is to determine the cause of defective phagocytosis in advanced lesions. Understanding the switch between the beneficial versus detrimental consequence of macrophage apoptosis, and whether the switch is driven by a defect in efferocytosis, remain crucial areas of future research into the cause of acute atherothrombotic vascular disease.

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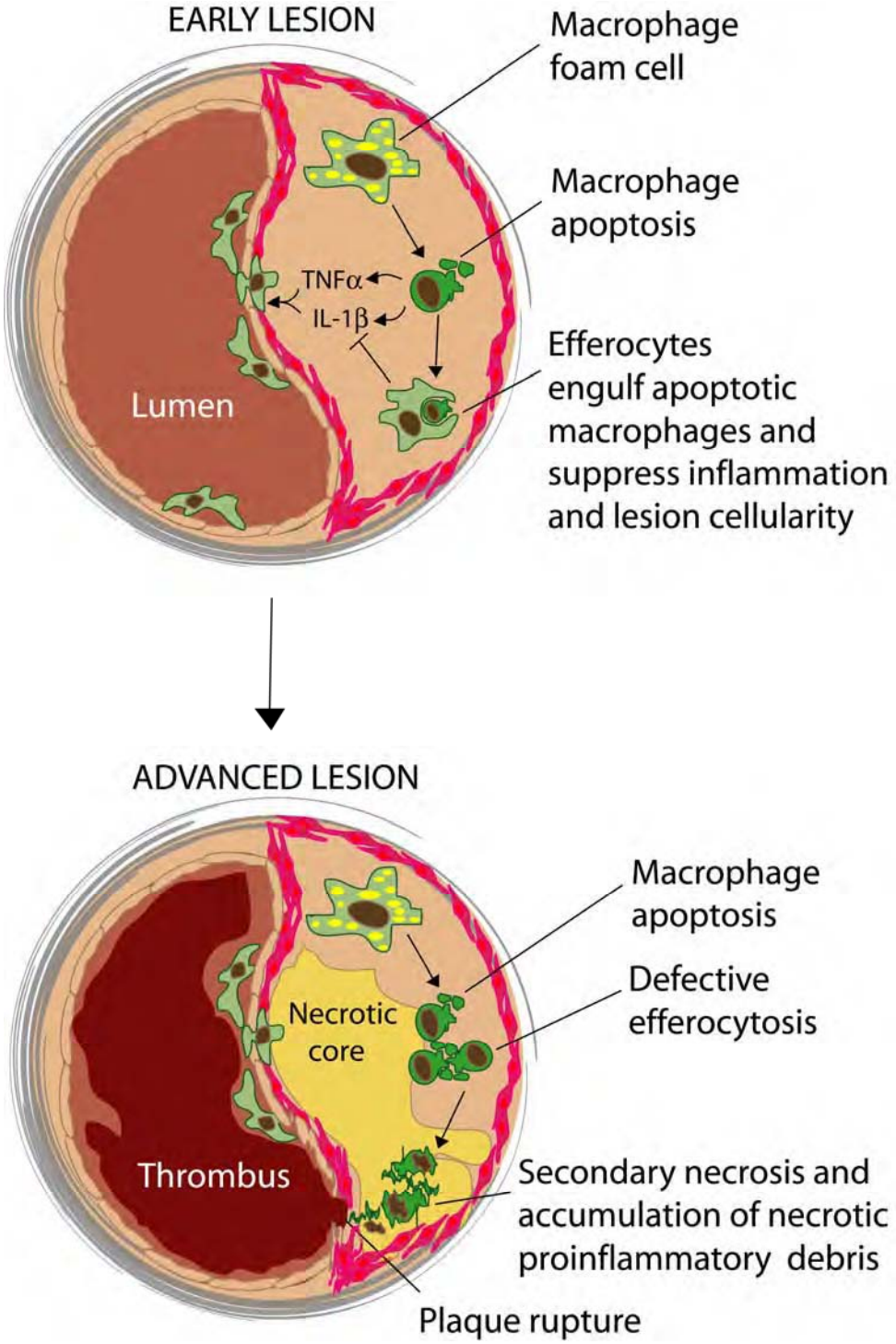
FIGURE LEGENDS

Figure 1: Model of the functional consequences of macrophage apoptosis in early and advanced lesions. In early lesions, monocytes surveying the vascular wall are recruited to the developing plaque. The monocytes differentiate into macrophages in areas where modified and remnant lipoproteins are retained in the extracellular matrix. Macrophages become foam cells by ingesting lipoproteins and storing these lipids in droplets. The engorged foam cells secrete a variety of proinflammatory cytokines and then eventually undergo apoptosis. Rapid efferocytic clearance of the apoptotic cells leads to suppression of the proinflammatory response. The overall effect is a reduction in lesion cellularity and size. In advanced lesions the apoptotic macrophages are not efficiently cleared by efferocytosis. The apoptotic macrophages that accumulate eventually undergo secondary necrosis. The buildup of necrotic debris promotes inflammation, plaque instability, and acute thrombosis.

Figure 2: Potential inducers of macrophage apoptosis in atherosclerotic lesions. Macrophage apoptosis can be triggered by a variety of factors that work alone or, most likely, in combination to trigger macrophage death. The buildup of endogenous ligands that are recognized by SRA, CD36, and TLR's trigger both a proinflammatory and apoptotic response during ER stress. See text for details.



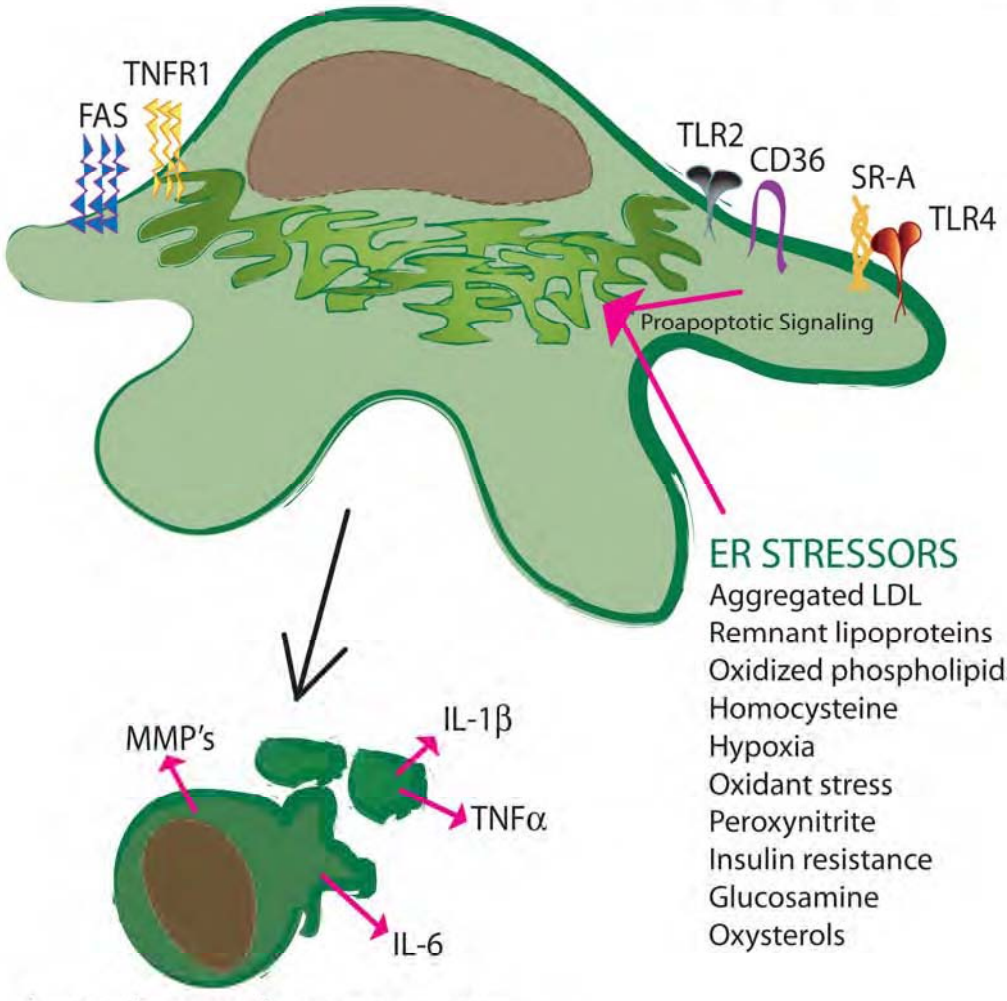
Seimon and Tabas, Figure 1

APOPTOTIC INDUCERS

TNFα
Fas ligand
Growth factor withdrawal
Oxysterols

PRR LIGANDS

Modified LDL
β-amyloid
Glycated collagen
AGE's
Oxidized phospholipids
Endotoxins and bacterial products



ER STRESSORS

Aggregated LDL
Remnant lipoproteins
Oxidized phospholipids
Homocysteine
Hypoxia
Oxidant stress
Peroxynitrite
Insulin resistance
Glucosamine
Oxysterols

Apoptotic macrophages are a source
of proinflammatory cytokines and proteases

Seimon and Tabas, Figure 2